



Edited by
Kostas Bourtzis
Thomas A. Miller

INSECT SYMBIOSIS

Volume 3



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INSECT SYMBIOSIS

Volume 3

CONTEMPORARY TOPICS in ENTOMOLOGY SERIES

THOMAS A. MILLER Editor

Insect Symbiosis

Edited by Kostas Bourtzis and Thomas A. Miller

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Cover: The genome sequences of the *Buchnera aphidicola* symbionts of *Schizaphis graminum* and *Acyrtosiphon pisum* have been determined. A comparison of the two genomes shows that no rearrangements or gene acquisition have occurred in the past 50 to 70 million years, despite the high levels of nucleotide-sequence divergence. This is the first time that whole-genome evolution for microbes has been calibrated with respect to time. The analysis has shown that *B. aphidicola* have the most stable genomes characterized to date. (Photograph courtesy of Ola Lundström, Department of Molecular Evolution, Uppsala University, Uppsala, Sweden.)

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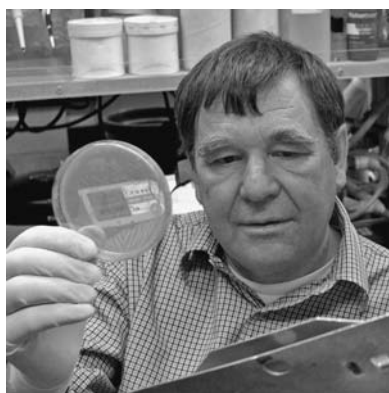
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Dedication to Paul Baumann



I still remember very clearly my first conversation with Paul Baumann. He had phoned to ask if I might be interested in collaborating on a study on the bacterial endosymbionts of aphids; the year was 1990. His excitement was infectious. I had read much of Paul Buchner's book as a graduate student but had never considered working on symbionts and knew almost nothing of microbiology.

During the earlier parts of his career, Paul had already contributed major work on topics related to marine bacteria and *Bacillus* pathogens of mosquitoes. Now he saw the possibility of understanding diversity of noncultivable symbionts in a firm molecular phylogenetic context and, further, to use molecular methods to explore symbiont functions. When I met him, Paul already had produced the first sequence-based characterization of obligate endosymbionts, work that resulted in the unexpected finding that the primary endosymbionts, which he named *Buchnera aphidicola*, were rather closely related to *Escherichia coli* (Unterman and Baumann, 1989). He realized that a close relationship to *E. coli* was a godsend for further investigations on functional capabilities of the symbiont. At that time, few genes were sequenced for any organisms, and homology to the best-studied model organism made it more likely that symbiont genes could be identified, cloned, and sequenced as a step toward understanding functional capabilities of these noncultivable organisms. Displaying a habit that I was to discover was usual, Paul had done exhaustive reading of the relevant literature, including papers on nutrition of aphids that suggested a role of symbionts in nutrition. By bringing new molecular data to the understanding of symbionts, he opened a new frontier in symbiosis studies and in the broader understanding of how microbes interface with multicellular eukaryotes.

Even when his results were groundbreaking, and even though his own enthusiasm was always immense, Paul had been consistently understated in how he presented results in publications and in speaking. He maintained an old-fashioned faith in his fellow scientists: that they would recognize an important result on its own merits and that "hype" would only distract (or insult) worthy readers. The first paper on codiversification of *Buchnera* and aphids (Munson et al., 1991) was a very important one, the first of many studies from many investigators to show that the phylogeny of bacteriome-associated symbionts mirrors that of their hosts. These matching phylogenies of host and symbiont are the strongest evidence for the antiquity of the associations, implying that the symbionts descend from an ancient infection predating the origin of the host group (~150 My in the case of aphids).

But this result was really a single sentence in the discussion of a paper in *Journal of Bacteriology*, without a figure. Often, this important result is attributed to a later paper (Moran et al., 1993), which expanded it and added a calibrated rate of evolution for the bacteria.

Paul, working with his wife, Linda, and others in his group at Davis, continued to methodically clone and sequence fragments identified on the basis of homology to *E. coli*, focusing on the genes underlying the biosynthesis of essential amino acids. The size of the *Buchnera* genome had earlier been claimed to be fivefold greater than that of *E. coli*, so, when the age of genomics dawned in 1995 with the full genome sequence of *Haemophilus influenzae*, *Buchnera* did not seem an obvious target for genomic sequencing (this was later shown to be wrong, with the *Buchnera* genome only one-seventh that of *E. coli*). By the time the first genome of *Buchnera* was published in 2000, Paul had characterized many *Buchnera* genes, including those encoding numerous enzymes in amino acid biosynthesis, and he had discovered that the genes underlying the rate-limiting step of tryptophan synthesis were amplified and located on an unusual plasmid, an apparent adaptation of the symbiont to better supply its host with this required nutrient (Lai and Baumann, 1994).

While understanding symbiont function has been one of Paul's primary aims, exploring unknown diversity has been the other. Between 1990 and 2005, he catalogued, sometimes collaborating with me, the remarkable assortment of symbiont types within most of the major insect groups related to aphids, including whiteflies, psyllids, and mealybugs (summarized in Baumann, 2005).

Part of why he was able to put the study of symbiosis on a firm foundation using molecular biology was that he avoided vague speculations and adhered to meticulous standards for data collection and quality. He believed in getting the facts right. As an illustration, when he once discovered minor errors in DNA sequences previously submitted to GenBank (at a time when bases were still being called manually from autorads of sequencing gels and when unresolved and erroneous positions were frequent in sequence databases), he resequenced the same templates and corrected the submissions, even though the papers were already published and even though there were no changes to the conclusions. His aversion to sloppiness was a force helping to set standards for the emerging field of molecular biology of symbiosis.

Although a perfectionist, his science was fueled by a sense of adventure and an attraction to exploring the unknown. In fact, much was unknown, including much that, thanks in large part to his efforts, is now known and taken for granted by students and others just starting in the field of symbiosis studies. It is now widely accepted that symbionts extend deep into the evolutionary history of insects and other eukaryotes and that we can explore their functions using knowledge of genetics and molecular biology derived from model systems. Paul and Linda's work is a major reason for these insights and others.

Paul and Linda retired in 2005. He is pursuing long-term interests in music, photography, and history (all areas in which he has remarkable expertise and characteristic enthusiasm). Symbiosis research, now hugely expanded compared to when he started, owes much to his pioneering vision and high standards.

Nancy A. Moran
University of Arizona

Some Citations from Paul Baumann's Work

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Preface to Insect Symbiosis, Volume 3

Volume 1 of this series contained a foreword by the noted science writer Carl Zimmer and was dedicated to Professor Frank Richards for his pioneering work in paratransgenesis and symbiotic control. The second volume had a foreword from Lynn Margulis, the noted symbiosis pioneer, and an obituary of Professor Hajime Ishikawa, a pioneer in insect symbiosis, written by his outstanding student Takema Fukatsu. This third volume has this preface and a dedication to the pioneering work of Paul Baumann in insect symbiosis, written by his long-time collaborator, Nancy Moran.

The field of insect symbiosis continues to grow. As we write this, Kostas leads a consortium of European colleagues at the inaugural meeting of the symbiosis group to initiate funding in the European Union for research on symbiosis to discover new tools for pest and disease control in agriculture and medicine (Brussels, Belgium, March 2008), and Thomas helps organize a meeting called by the Ministry of Agriculture, Rabat, Morocco, aimed at finding new tools of biotechnology for control of desert locust, *Schistocerca gregaria*. Symbiosis offers one bright hope for a breakthrough in this historically difficult pest problem.

The present volume contains reports from outstanding laboratories across the field of insect symbiosis and includes work suggesting or hinting at practical applications in mosquitoes, tephritids, and termites. There are more examples in this volume of the influence of symbionts on the biology of insects.

About the Editors

Kostas Bourtzis, Ph.D., is Associate Professor of Molecular Biology and Biochemistry in the Department of Environmental and Natural Resources Management, University of Ioannina, Greece. His research interests include *Wolbachia*-mediated cytoplasmic incompatibility in *Drosophila*, agricultural insect pests and disease vectors; genetic manipulation of *Wolbachia*; molecular mechanism of cytoplasmic incompatibility; *Wolbachia* genomics; and the use of endosymbiotic bacteria including *Wolbachia* as a tool for the development of new, environmentally friendly approaches for the control of arthropods of medical and agricultural importance. His group has recently shown that *Wolbachia*-induced cytoplasmic incompatibility can be used as a means to suppress insect pest populations.

Thomas A. Miller, Ph.D., is Professor of Entomology at the University of California, Riverside, where he teaches insect physiology and insect toxicology. A B.A. in physics influenced his earlier work in insect neurophysiology, including the development of electronic transducers for measuring small muscle forces. This was followed by contributions to mode of action of insecticides and developing methods of measuring resistance in cotton pests, which again involved the development of unique electromechanical devices to record insect activity in the field. Dr. Miller led the successful effort to genetically transform the pink bollworm and provided the opportunity to employ a conditional lethal strain for use in area-wide eradication. All of these achievements were done with students, colleagues, and collaborators. Most recently, Dr. Miller has been seeking ways of applying principles of biotechnology to pest and disease control, again with a host of collaborators.

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chapter one

Insect symbionts and molecular phylogenetics

Václav Hypša and Eva Nováková

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The significance of molecular phylogenetics for insect symbiont research

Investigation into prokaryote evolution is one of the areas of biology most deeply affected by the advent of molecular phylogenetics (Pace, 1997). In the field of insect–bacteria symbiosis, this new approach not only generated an immense amount of knowledge, but also fundamentally changed the whole framework of this field: bacterial symbionts are now mostly viewed and analyzed in terms of *phylogenetic lineages*. This situation contrasts with the premolecular era, when morphological and functional characterization provided the only base for symbiont classification. Until the late 1980s, studies on insect bacterial symbionts were either purely descriptive or focused on functional rather than evolutionary aspects (Buchner, 1965; Hill and Campbell, 1973; Griffiths and Beck,

1974; Nogge, 1978; Ishikawa, 1982; Eberle and McLean, 1983); when tackling evolutionary issues at all, they were largely speculative and could rely on only indirect evidence (Buchner, 1965). The new phylogeny-based way of looking at the symbionts allowed for addressing many previously inapproachable issues, such as the origin of various symbiotic lineages in frame of the whole prokaryote diversity, degree of vertical versus horizontal transfers, or continuous acquisitions and losses of various symbiotic lineages by their hosts. On the other hand, this new approach introduced into the symbiosis field a variety of methodological problems related to the intricacy of phylogenetic inference and coevolutionary reconstructions.

Within less than two decades, a substantial number of analyses have been performed and published. From a formal point of view, this process resulted in identification and taxonomic description of many symbiotic lineages (Table 1.1). More importantly, several interesting patterns began to emerge from the accumulated data. In Figure 1.1, we provide a rough picture of insect–symbiont diversity mapped onto a phylogenetic background. Because there is little agreement on the overall phylogenetic arrangement of bacterial symbionts, this topology is meant as a reference scheme for the following discussions rather than as an attempt to achieve consensus of the current views. Despite the high degree of uncertainty and subjectivity, it demonstrates several important facts. At broad phylogenetic scale, the expected polyphyly of bacterial symbionts was well established. It is now clear that symbiotic lineages have originated multiple times within many groups of prokaryotes. On the other hand, hot spots giving rise to multiple symbiotic lineages can be identified within some taxa. For example, many of the major symbiotic lineages seem to stem from a particularly prominent spot within γ -Proteobacteria (the node P in Figure 1.1). This issue, however, remains highly controversial and will be discussed later.

Another outcome of phylogenetic studies is the apparent versatility of bacterial lineages. Closely related bacteria can rapidly evolve into a highly specialized mutualist as well as parasites/commensals loosely associated with their hosts (nodes P, A, and S in Figure 1.1 show such diverse groups). This versatility has even been experimentally demonstrated by functional replacement of obligate long-established mutualists with other symbiotic bacteria (Koga et al., 2003). Several recent studies indicate that at least some of the symbiotic lineages previously believed to have originated by only a single and unique transition from a free-living bacterium to symbiosis, may in fact represent abundant and widely distributed symbionts (e.g., the genera *Sodalis* and *Arsenophonus*). An increasing tempo of identification and characterization of new symbiotic lineages will certainly lead to an even more complex picture and therefore higher demands on coevolutionary reconstruction and its evolutionary interpretation. Consequently, the methods originally restricted to parasitological research, such as tree-based and data-based coevolutionary reconstructions, have begun to penetrate the field of symbiosis.

In this chapter, we highlight the main questions and methodological problems that arose from the current view on insect–bacteria symbiosis. In the first section, we show that the phylogenetic information accumulated during the last years of molecular research has changed some of the previous conceptions of the field and puts insect–bacteria symbiosis into an entirely new perspective. In this context, we highlight the importance of unprejudiced and rigorous interpretation of phylogenetic relationships for evolutionary inferences. In the following sections, we focus on two major methodological problems, namely, the inference of phylogenetic signal from degenerated symbiotic genomes and the reconstruction of coevolutionary history between insects and their symbionts.

Table 1.1 List of Symbiotic Bacteria Included in the Tree in Figure 1.1

Taxon Designation	Classification	Host	References
<i>Arsenophonus</i> sp.	Gamma	Various arthropods	(Ghera et al., 1991; Hypša and Dale, 1997; Thao and Baumann, 2004b)
<i>Baumannia cicadellinica</i>	Gamma	Various spp. of sharpshooters	(Moran et al., 2003)
<i>Bemisia tabaci</i> *	Gamma	**	(Zchori-Fein and Brown, 2002)
<i>Blochmania</i> sp.	Gamma	Various spp. of carpenter ants	(Schroder et al., 1996; Sauer et al., 2000)
<i>Buchnera aphidicola</i>	Gamma	Aphids	(Munson et al., 1991b)
<i>Carsonella ruddii</i>	Gamma	Psyllids	(Spaulding and von Dohlen, 1998; Clark et al., 2001; Nakabachi et al., 2006)
<i>Cimex lectularius</i> *	Gamma	**	(Hypša and Aksoy, 1997)
<i>Columbicola columbae</i> *	Gamma	**	(Fukatsu et al., 2007)
<i>Craterina malbae</i> *	Gamma	**	(Nováková and Hypša, 2007)
<i>Euscelidius variegatus</i> *	Gamma	**	(Campbell and Purcell, 1993)
<i>Hamiltonella defensa</i>	Gamma	Various spp. of aphids and psyllids	(Moran et al., 2005a)
<i>Hematomizus elephantis</i> *	Gamma	**	(Hypša and Křížek, 2007)
<i>Hematopinus</i> sp.*	Gamma	**	(Hypša and Křížek, 2007)
<i>Ishikawaella capsulata</i>	Gamma	Various spp. of stinkbugs	(Hosokawa et al., 2006)
Mealybugs A*	Gamma	Various spp. of mealybugs	(Thao et al., 2002)
Mealybugs C	Gamma	Various spp. of mealybugs	(Thao et al., 2002)
Mealybugs D*	Gamma	Various spp. of mealybugs	(Thao et al., 2002)
Mealybugs E*	Gamma	Various spp. of mealybugs	(Thao et al., 2002)
<i>Nardonella</i>	Gamma	Various spp. of weevils	(Lefevre et al., 2004)
<i>Polyplax</i> sp.*	Gamma	**	(Hypša and Křížek, 2007)
<i>Portiera aleyrodidarum</i>	Gamma	Whiteflies	(Thao and Baumann, 2004a)
<i>Pseudolynchia canariensis</i> *	Gamma	**	(Dale et al., 2006)

Continued.

Table 1.1 List of Symbiotic Bacteria Included in the Tree in Figure 1.1 (*Continued*)

Taxon Designation	Classification	Host	References
Psyllids 1*	Gamma	Various spp. of psyllids	(Thao et al., 2000a)
Psyllids 2*	Gamma	Various spp. of psyllids	(Thao et al., 2000a)
<i>Regiella insecticola</i>	Gamma	Various spp. of aphids	(Moran et al., 2005a)
<i>Riesia pediculicola</i>	Gamma	Primate lice	(Allen et al., 2007)
<i>Serratia symbiotica</i>	Gamma	Various spp. of aphids	(Moran et al., 2005a)
<i>Sitophilus</i> sp.*	Gamma	**	(Lefevre et al., 2004)
<i>Sodalis glossinidius</i>	Gamma	<i>Glossina</i> sp.	(Dale and Maudlin, 1999; Dale et al., 2001; Toh et al., 2006)
<i>Trichobius</i> sp.*	Gamma	Various spp. of bat flies	(Trowbridge et al., 2006)
Weevils*	Gamma	Various spp. of weevils	(Lefevre et al., 2004)
<i>Wigglesworthia glossinidia</i>	Gamma	<i>Glossina</i> sp.	(Aksoy, 1995)
<i>Asaia</i> sp.	Alpha	<i>Anopheles stephensi</i>	(Favia et al., 2007)
<i>Bartonella</i> sp.	Alpha	Various insect species	(Reeves et al., 2005)
<i>Diaphorina citri</i> A*	Alpha	**	(Subandiyah et al., 2000)
<i>Rickettsia</i> sp.	Alpha	Various arthropods	(Sakurai et al., 2005; Perotti et al., 2006)
<i>Wolbachia pipientis</i>	Alpha	Various arthropods and nematodes	(Yen and Barr, 1971; O'Neill et al., 1992)
<i>Diaphorina citri</i> B*	Beta	**	(Subandiyah et al., 2000)
<i>Tremblaya princeps</i>	Beta	Mealybugs	(Thao et al., 2002)
<i>Adonia variegata</i> *	Bacteroidetes	**	(Hurst et al., 1999)
<i>Blattabacterium cuenoti</i>	Bacteroidetes	Various spp. of cockroaches and termites	(Bandi et al., 1995; Lo et al., 2003)
<i>Cardinium</i> sp.	Bacteroidetes	Various arthropods	(Zchori-Fein and Perlman, 2004; Marzorati et al., 2006)
<i>Coleomegilla maculata</i> *	Bacteroidetes	**	(Hurst et al., 1997)
<i>Cryptococcus ulmi</i> *	Bacteroidetes	**	(Gruwell et al., 2007)
<i>Icerya</i> sp.*	Bacteroidetes	**	(Gruwell et al., 2007)
<i>Sulcia muelleri</i>	Bacteroidetes	Various spp. of cicadas, leafhoppers, spittlebugs, treehoppers	(Moran et al., 2005b)

Continued.

Table 1.1 List of Symbiotic Bacteria Included in the Tree in Figure 1.1 (Continued)

Taxon Designation	Classification	Host	References
<i>Uzinura diaspidicola</i>	Bacteroidetes	Various spp. of armored scale insects	(Gruwell et al., 2007)
<i>Zigia versicolor</i> *	Bacteroidetes	**	(Zchori-Fein and Perlman, 2004)
<i>Spiroplasma</i> sp.	Firmicutes	Various arthropods	(Tsuchida et al., 2006)

Note: P-symbionts are printed in bold. The alpha, beta, and gamma terms stand for individual subdivisions of Proteobacteria. As in Figure 1.1, the taxa are designated by either the names of symbiotic bacteria or those of the host taxa labeled with an asterisk. For the latter case, asterisks are used within the Host column instead of repeating the host name.

P-symbionts and S-symbionts: old terms in a new phylogenetic framework

The “primary” (P) and “secondary” (S) symbionts are among the most firmly established terms in insect symbiosis research. They originated early in the prephylogenetic period of this field (Buchner, 1965) from a highly simplified view: in addition to evolutionary original and nutritionally essential bacteria inhabiting specialized host cells (P-symbionts), some insects carry incidental infections with dispensable or even deleterious bacteria (S-symbionts). Although these terms are frequently used in the literature, their exact meaning remains vague. Based on the above demarcation, several typical traits are usually attributed to these two categories. P-symbionts are large bacteria restricted to specialized host cells (bacteriocytes), inherited exclusively by vertical mode and cospeciating with their host. In contrast, S-symbionts can invade various cells of the host and are often transferred horizontally among unrelated host species or higher taxa.

With the growing diversity of characterized symbionts, this conception is still more difficult to apply and the terms are used with considerable uncertainty. This fact was succinctly stated in the exhaustive review on P-symbionts by Paul Baumann:

In contrast to this relatively well-defined association between the P-endosymbionts and insects, the association between S-symbionts and insects is currently not amenable to a simple definition, because these organisms form a heterogeneous group with respect to location in the insect and possibly in their function (Baumann, 2005).

For example, when multiple symbiont lineages occur in an individual host, some of the bacteria designated as S-symbionts can display traits very similar to P-symbionts, including congruent phylogeny with the host (Thao et al., 2002). The same uneasiness with this concept has led Takiya et al. (2006) to coin the term *coprimary* symbionts for cases where “two or more symbionts are obligate and ancient bacteriome-associates.” Moreover, many phylogenetic analyses demonstrate that there is no fundamental division between these two types of symbionts. As can be seen in Figure 1.1, bacterial lineages display remarkable versatility and can rapidly adapt to various kinds of symbiosis. This dynamic view of symbiont evolution is further supported by additional observations, such as multiple infections and/or symbiont replacements (Lefevre et al., 2004; Takiya et al., 2006; Wu et al., 2006). Experimental evidence of the feasibility of such replacements in highly specialized

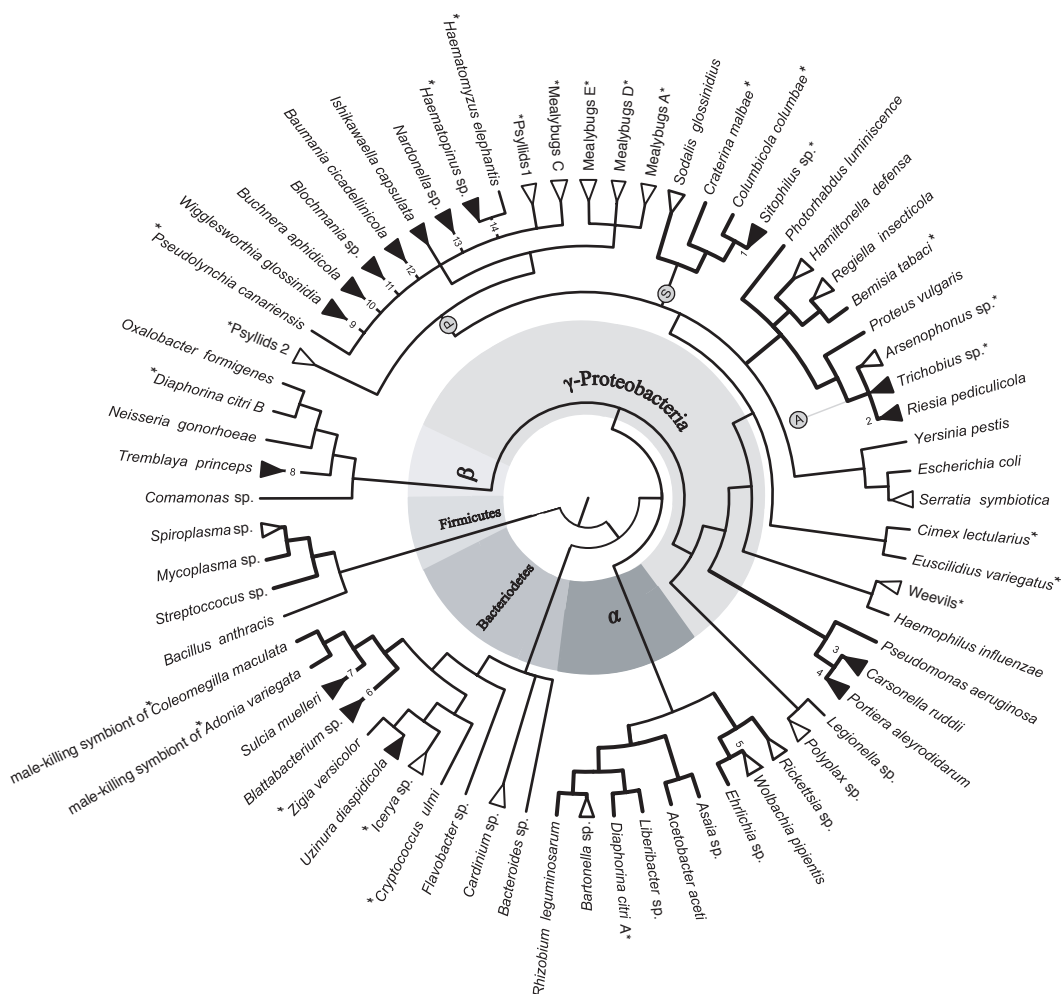


Figure 1.1 Phylogenetic positions of bacteria living in symbiosis with insects. The tree was built using rather subjective criteria (see the text), using published phylogenetic studies. The labels in the tree present either the names of symbiotic bacteria or those of the host taxa (marked with an asterisk). Free-living bacteria are printed in gray. Bold lines designate the relationships for which general consensus exists in the literature. The triangles symbolize monophyletic diversified groups of symbionts described from more than a single host species (solid triangle: lineage phylogenetically congruent with the host). P: putative origin of multiple symbiotic lineages including several of major P-symbionts; S and A designate the nodes discussed in the text. Information on the host taxa and source literature are provided in Table 1.1. Time estimates for the numbered nodes are listed in the Table 1.2.

associations comes from a study demonstrating the capacity of S-symbionts to take over a nutritional role to replace the eliminated P-symbionts (Koga et al., 2003).

As in many other ecologically rooted categories (i.e., parasites vs. commensals, parasite vs. parasitoid), it is unlikely that the diversity of symbiotic bacteria can be fitted into a simple and unequivocal terminological framework. However, to make the following discussions as transparent as possible, we use the P-symbiont and S-symbiont terms in their

most conservative meaning. We set aside the chronological question of the associations' origin and reserve the term P-symbiont for the clearly coevolving mutualists, such as *Buchnera* or *Wigglesworthia*. All other types of symbionts are referred to as S-symbionts, with full awareness of the ecological and functional heterogeneity of this category.

Composition of symbiotic fauna: transitions, losses, and replacements

It has been generally supposed that the compensation for missing nutrients by symbionts is a prerequisite of host survival and adaptive radiation for some feeding strategies. The logical consequence in such cases would be a unique origin and perpetual coevolution between the host and its symbiont. The first phylogenetic descriptions were indeed in accordance with such a model: both aphids and tsetse flies form well-defined groups with specific life strategies and harbor unique monophyletic lineages of symbionts (Munson et al., 1991a; Chen et al., 1999). However, an increasing amount of molecular evidence shows that this strict coevolutionary view has to be relaxed. Multiple infections with distant P-symbionts, losses, and/or replacements often shape insect–bacteria associations. Even the host groups nutritionally dependent on typical P-symbionts can harbor an assemblage of phylogenetically distant symbionts, which have arisen through a series of acquisitions/losses and display a mosaic of different coevolutionary histories. Such a decoupled life strategy and symbiosis history can be demonstrated by the composite P-symbiont fauna of two groups of hematophagous insects, Anoplura and Hippoboscoidea.

As obligatory hematophages, sucking lice (Anoplura) conform to a widely accepted definition of insects dependent on bacterial symbionts (Douglas, 1989) and their nutritional dependence has even been experimentally demonstrated by elimination of symbionts (Aschner, 1934; Puchta, 1955). Because hematophagy is clearly a universal feeding strategy that originated in the common ancestor of extant anoplurans, one should expect to see an uninterrupted chain of louse-symbiont cospeciations. Such a picture is indeed found when louse-symbiont associations are studied at low phylogenetic levels; symbiotic bacteria of the genus *Riesia* have been shown to mirror phylogenetic relationships of their host genera *Pediculus* and *Phthirus* (Allen et al., 2007). However, within the frame of the whole order Anoplura, P-symbionts form an apparently polyphyletic assemblage exceeding even the borders of Enterobacteriaceae (Hypša and Křížek, 2007). Considering their nutritional significance, the polyphyletic nature of louse P-symbionts, contrasting to the perfect coevolution of *Buchnera*-aphid, may seem unexpected. Remarkably, a similar situation is encountered in yet another hematophagous taxon, the dipteran superfamily Hippoboscoidea. Within this group, the tsetse fly–*Wigglesworthia* symbiosis is often mentioned as a typical P-symbiont association analogous to the aphid–*Buchnera* system. However, this view is at least partly due to a long-lasting omission of three “unimportant” groups related to tsetse flies, the families Hippoboscidae, Nycteribiidae, and Streblidae. Only recently it was shown that the families Hippoboscidae and Streblidae carry P-symbiotic bacteria clustering within the genus *Arsenophonus*, at a position very distant from *Wigglesworthia* (Trowbridge et al., 2006).

Both Anoplura and Hippoboscoidea consist of obligatory hematophages and are likely to have differentiated from already hematophagous ancestors. Although the analogy with *Buchnera* would suggest an ancient establishment of symbiosis followed by a long coevolutionary history, both groups seem rather to have undergone multiple symbiont acquisitions, losses, and replacements. Although they provide a good example, sucking lice and hippoboscids are certainly not the only groups that underwent such a rich history of symbiotic associations. Many other insect taxa seem to harbor wealthy assemblages of

symbionts that have arisen from repeated acquisitions and losses (Lefevre et al., 2004; Takiya et al., 2006). As a result of such processes, the gross phylogenetic picture combines several different patterns of symbiotic lineages: (1) long-term coevolution between a P-symbiont and host; (2) multiple infections by different P-symbionts within a host; and (3) replacements of established symbiotic lineages. Each of these specific arrangements represents a different challenge to the phylogenetic reconstruction. For example, most of the typical P-symbiotic lineages can be well recognized as monophyletic groups and their coevolution with the host can be easily established (Munson et al., 1992; Schröder et al., 1996; Chen et al., 1999). The main difficulty rests in determining their position within bacterial phylogeny and is due to far-reaching modifications of their genomes. On the other hand, most S-symbionts retain sufficient phylogenetic information in their sequences to allow for reliable phylogenetic placement. However, it might be difficult to find a scenario reconciling their phylogeny with host distribution. These areas of phylogenetic uncertainty are further discussed in the two following sections.

P-symbionts: a touchstone of molecular phylogenetics

The most interesting and debated node in the whole tree of symbiotic bacteria is undoubtedly the putative origin of many symbiotic lineages within Enterobacteriaceae (Charles et al., 2001). In Figure 1.1, this node is presented in its “maximal” version, encompassing several major P-symbionts and many minor lineages (node P). However, in the published studies, the whole issue has mostly been addressed by analyzing phylogenetic relationships of the two most popular groups, *Buchnera* and *Wigglesworthia*. Although retrieved by a majority of phylogenetic studies, the monophyly of the symbiotic cluster containing these two P-symbiont lineages has been legitimately questioned. This doubt arises because the genomes of P-symbionts meet typical conditions leading to phylogenetic artifacts. Compared to their free-living relatives, P-symbiotic lineages display remarkably high frequency of AT in their sequences. This bias is considered one of the most significant symptoms of genome degradation in symbiotic bacteria. For the first time it has been detected within 16S rDNA and is usually attributed to relaxed selection together with Muller’s ratchet occurring in small asexual populations (Moran, 1996; Heddi et al., 1998; Lambert and Moran, 1998; but see Itoh et al., 2002, for alternative explanation). The statistical significance of this compositional shift was later confirmed by Haywood-Farmer and Otto (2003).

Because the difference in nucleotide composition among the lineages seriously violates assumptions implemented in the majority of phylogenetic methods, it results in more or less predictable artifacts. Currently, the most common approach to this problem is an employment of techniques designed to eliminate or at least suppress the effect of compositional heterogeneity. Several such methods have been proposed and this area is undergoing fast advancement. An alternative approach does not rely on extraction of phylogenetic signal by tuning the assumptions to (supposedly) real evolutionary process, but rather to extend the dataset and/or to find alternative sources of information. Genome-wide concatenation of protein-coding genes and extraction of a phylogenetic signal from genome structure are such techniques.

Nonhomogeneous models

Most P-symbiont studies have used phylogenetic methods implementing standard models of molecular evolution, which are based on stochastic processes with two main implicit assumptions: homogeneous base composition and constant substitution rates. Due to

these assumptions, the evolutionary processes can be modeled and analyzed using the time-reversible Markov chain model as the methodological basis. Consequently, if any force directs substitution processes, the assumption of time-reversibility becomes violated. A typical, well-known example of such selection-driven change is the compositional difference between 16S rRNA genes of thermophilic and mesophilic bacteria. When analyzed in the context of thermophiles, the mesophilic bacteria *Deinococcus* and *Bacillus* cluster as sister groups, in contrast to strong evidence for their polyphyly. This conflict has been repeatedly attributed to convergent selection-driven evolution of thermophiles toward a GC-rich genome (Mooers and Holmes, 2000; Foster, 2004). A similar effect can be seen in the AT-rich sequences of symbiotic bacteria. Particularly in 16S rDNA analysis, this phenomenon can play a crucial role, because long stretches within the transcribed rRNA loops can accommodate an enrichment of AT residues.

This problem does not have any simple solution. Initial attempts to cope with it relied on distance calculations eliminating the effect of composition heterogeneity, particularly the paralinear (Logdet) method (Lake, 1994; Lockhart et al., 1994) or alternative distance formula suggested by Galtier and Gouy (1995). However, the distance methods are generally considered an inferior phylogenetic tool compared to the maximum parsimony (MP), maximum likelihood (ML), or Bayesian analysis. It is therefore understandable that the nonhomogeneous approach was soon introduced into the maximum likelihood framework.

The model developed by Yang and Roberts (1995) extended the well-known HKY85 (Hasegawa et al., 1985) substitution model by introducing different compositional parameters for each tree branch. Although this algorithm is in principle capable of dealing with nonhomogeneous sequences, this model is too parameter rich and thus computationally demanding. Moreover, the necessity to estimate parameters from the data is a potential source of topological distortions. To overcome these difficulties, Galtier and Gouy (1998) simplified the model by replacing the HKY basis with T92 (a single parameter for G + C) (Tamura, 1992). It was only this new version of the nonhomogeneous model that was subsequently used to test the monophyly/polyphyly of the P-symbiotic lineages (Herbeck et al., 2005). This study brought the first strong evidence favoring P-symbionts polyphyly. However, it has not settled the issue at all. On the contrary, several authors expressed their dissent with the polyphyletic view and tried to prove the opposite.

The main problem is that while there is no doubt about the superior performance of nonhomogeneous model(s) in some particular cases, it may be extremely difficult to predict their behavior for various matrices and datasets. Indeed, selection of a proper model is one of the very central issues of ML methodology. A well known property of evolutionary models is that their predictive power decreases with additional parameters (Posada and Buckley, 2004; Steel, 2005). The nonhomogeneous model applied to the P-symbiont phylogeny uses a free compositional parameter(s) on each branch, which may rapidly lead to the over-parameterization of the analysis with the increasing number of branches in the tree. Ultimately, this property is a reason why the nonhomogeneous technique may not be particularly suitable for solving the P-symbionts issue. To decrease the complexity of the nonhomogeneous model, Foster (2004) suggested that instead of introducing many free parameters along a tree, an application of only a few vectors of composition is sufficient to handle compositional changes. He used the aforementioned thermophile problem to test this method and showed that it can indeed be solved by introducing only two vectors. To find the optimal solution, he employed Bayesian analysis to test the fit of the nonhomogeneous model to data. Lately, the nonhomogeneous models are being further developed in several different directions (Blanquart and Lartillot, 2006; Gowri-Shankar and Rattray,

2007). None of these new Bayesian-based methods, optimizing the number of parameters, have so far been used to address the P-symbiont issues. However, rapid development of the techniques extracting phylogenetic signal from heterogeneous sequences indicates that it would be premature to draw any conclusion on P-symbionts monophyly/polyphyly from the analyses that have been reported.

Multigene approach

Leaving aside the question of a “proper” algorithm, it should be admitted that both monophyletic and polyphyletic arrangements are only poorly supported by the 16S rRNA-derived signal. In other words, the 16S rRNA gene, the most frequently used marker in bacterial phylogeny, is incapable of solving the relationships among P-symbionts. This observation is not surprising because such insufficiency of rRNA genes is frequently observed at various phylogenetic levels in many groups of organisms. Various protein-coding genes have been used as an alternative source of phylogenetic information (Degnan et al., 2004; Casiraghi et al., 2005; Moran et al., 2005a; Baldo et al., 2006; Fukatsu et al., 2007). Although they can yield better phylogenetic resolution at some particular nodes in dependence of their evolutionary tempo, they do not provide any fundamental advantage if used in single-gene matrices.

The only way of overcoming the lack of reliable information is an extension of the dataset with additional sequences and employment of the multigene approach. Although seemingly simple and straightforward, the method of adding new genes is not free of potential troubles. The typical bacterial genome is a flexible assemblage of genes undergoing frequent structural changes (Snel et al., 2005). Some of these processes may hinder selection of suitable universal markers among hundreds of possible candidates. For example, loss of genes leads to absence of a given phylogenetic marker in some bacterial lineages. This situation may be particularly frequent in symbiotic bacteria that undergo rapid and dramatic loss of many genes; substantial reduction of genome size can be seen in all of the completely sequenced genomes of P-symbionts (Shigenobu et al., 2000; Akman et al., 2002; Gil et al., 2003; Nakabachi et al., 2006; Wu et al., 2006) and has been observed even in the presumably young symbiotic lineage *Sodalis glossinidius* (Toh et al., 2006). Moreover, different nutritional constraints in various host–symbiont associations lead to differential preservation/loss of various sets of genes in different symbionts. Thus, on their hypothetical pathways from free-living bacterium to highly specialized symbionts, *Buchnera* and *Wigglesworthia* reduced their genomes to approximately 583 and 621 coding genes, respectively (Shigenobu et al., 2000; Akman et al., 2002); only 69% of these genes are shared by both lineages (Akman et al., 2002). Similar functional complementarity between two different symbiotic genomes, although not based on complete genome sequences, was recently reported for the genera *Sulcia* and *Baumannia* (Wu et al., 2006). If such small genomes are to be analyzed together with free-living bacteria, genes have to be identified that are present in all of the included genomes. To make the situation even more complicated, successful identification of homologous genes is only one prerequisite, but does not itself guarantee a consistency of phylogenetic signal. At least two additional processes may disturb phylogenetic reconstruction. Duplications are a known and much feared source of paralogs, which are further inherited during the speciation process. A random sampling of paralogs from different lineages during the phylogenetic analysis can be a source of serious topological inaccuracies. Finally, even worse phylogenetic inconsistencies may arise due to horizontal gene transfer (HGT), a process that introduces phylogenetically distant xenologs into bacterial genomes.

The significance of duplication and HGT for phylogenetic inference in bacteria has not been fully elucidated. Generally, it is supposed that duplications in prokaryotes are less deleterious than in eukaryotic organisms. By contrast, the HGT is often detected in bacteria and has sometimes even been considered as one of the main forces shaping bacterial genomes. However, current views on this issue are largely dependent on the methods used to estimate overall HGT frequency (Lerat et al., 2003; Susko et al., 2006; Doolittle and Baptiste, 2007). For example, a conservative view, with vertical inheritance playing a predominant role in the bacterial genome structure, has been voiced by Lerat et al. (2003). These authors assessed the overall compatibility of individual single-gene matrices with selected topologies. To achieve this, they postulated phylogenetic congruence as a null hypothesis and used the Shimodaira-Hasegawa test (Shimodaira and Hasegawa, 1999) to identify an HGT by its rejection. Comparing 13 genomes of γ -Proteobacteria, they showed that the universally present orthologs suffer only negligible frequency of HGT: of 205 genes included in the analysis, 203 produced mutually compatible topologies. When used for phylogenetic inference within a concatenate matrix, this respectable set of genes produced a monophyletic and well supported branch of *Buchnera* + *Wigglesworthia* that was preserved even after removal of the AT-rich codons. On the other hand, such a low level of HGT has recently been questioned by Susko et al. (2006). They adopted methods from functional genomics to visualize the congruency within the core gene set suggested by Lerat et al. (2003) and concluded that around 10% of the genes may have resulted from HGT. In their discussion, they further postulate that using congruence as a null hypothesis and searching for the significant incongruence necessarily leads to underestimation of the HGT level. A similar opinion about the considerable occurrence of HGT has also been reached by Doolittle and Baptiste (2007) using an entirely different source of evidence than phylogenetics. Considering the whole spectrum of HGT frequency estimates, stretching from almost zero (Ge et al., 2005) to more than 60% (Lerat et al., 2005; Dagan and Martin, 2007), it is hard to assess the possible effect of this phenomenon on the selection of a suitable set of phylogenetic markers.

Genome structure

Changes in nucleotide sequences are not the only trace evolutionary processes left on the genome. Various other characters such as gene fusion (Philippe et al., 2000; Stechmann and Cavalier-Smith, 2002), codon reassignment (Castresana et al., 1998), RNA secondary structure (Billoud et al., 2000; Swain and Taylor, 2003), or gene arrangement (Lavrov et al., 2004) originate by unique evolutionary events and may retain valuable phylogenetic information (Rokas and Holland, 2000). Although some of these “idiosyncratic” markers (Murrell et al., 2003) may be affected by the same processes as nucleotide sequence (e.g., codon bias, secondary structure), others are likely to be sequence-independent. The most popular source of such markers by far is the arrangement of genes within a genome.

Although this tool has only recently been “discovered” for bacterial phylogeny and used in a few studies, the idea itself is relatively old. The reason for its omission in bacterial phylogeny rests mainly in the computation demands connected to genome-wide analysis. Most studies relying on this method have focused on eukaryotes and dealt with mitochondrial genomes (Lavrov et al., 2004; Negrisolo et al., 2004; Larget et al., 2005; Podsiadlowski et al., 2007). Their results showed that, like other sources of phylogenetic data, gene order can be helpful to solve some particular problems (Lavrov et al., 2004), whereas it fails in others (Negrisolo et al., 2004). Among the few analyses performed on prokaryotes, some are of very low informative value with respect to P-symbiont issues, because they

did not include P-symbiotic bacteria at all (Comas et al., 2006) or contained only a single P-symbiotic lineage (Korbel et al., 2002). The only analysis that explicitly addressed the monophyly/polyphyly of P-symbionts introduced yet another solution into the repertoire of suggested scenarios by arranging *Wigglesworthia* and *Buchnera* in a paraphyletic manner with respect to several free-living bacteria (Belda et al., 2005).

Evolutionary implications of P-symbionts monophyly/polyphyly

With our current state of knowledge, the answer to the P-symbionts issue can only rest on personal opinion. Seen from this perspective, the complex analysis of Comas et al. (2007) appears to provide the most convincing evidence on the monophyly of at least *Blochmannia* + *Wigglesworthia* + *Buchnera*. Although the opposite conclusion was reached in two recent studies based on different methods (Belda et al., 2005; Herbeck et al., 2005), their results are not mutually compatible. Under the nonhomogeneous model, *Buchnera* and *Wigglesworthia* were placed at very distant phylogenetic positions (Herbeck et al., 2005), whereas in gene-order analysis they branched as closely related lineages in paraphyletic arrangement (Belda et al., 2005). Considering these topological differences and the highly experimental nature of the utilized methods, the results indicating the non-monophyly of P-symbionts should for the present be treated with caution.

However, regardless the methodological reliability of various published analyses, at least two circumstances make their evolutionary interpretation uncertain. First, these studies only used datasets from a few selected symbiotic lineages. Because, as a rule, the accuracy of phylogenetic inference depends on dataset completeness, an inclusion of additional symbionts might affect both topology and support of the tree. In Figure 1.1, we show all symbiotic lineages that were placed by some analysis into the P-symbiont cluster. To the best of our knowledge, no published study included all, or at least a majority, of these symbionts. In the current literature, there is an obvious bias toward the most popular symbionts, namely *Buchnera* and *Wigglesworthia*, while others are often neglected. At least with genome-wide studies this situation can be attributed to the limited availability of complete genomes. Second, and more important, even the final solution of the monophyly/polyphyly problem does not provide a definitive answer to the origin of P-symbiotic bacteria. Upon retrieving the monophyletic *Buchnera*-*Wigglesworthia* clade, Canbäck et al. (2004) suggested a common origin of these two lineages from a symbiotic ancestor. To support this view, they referred to similar results obtained by Lerat et al. (2003) in their broad phylogenomic analysis. However, no such strong statement was made in the latter study. On the contrary, the authors rightly stated that due to the lack of any gene common for *Buchnera* + *Wigglesworthia* but absent in other related bacteria, the issue of symbiosis origin cannot be effectively addressed.

Obviously, the monophyly of the P-symbiotic lineage does not necessarily imply a unique transition from a free-living bacterium. Considering the evolutionary distance among homopterans and dipterans, such a hypothesis would require an establishment of symbiosis extremely deep within the insect phylogeny, followed by a high frequency of secondary losses. This is not entirely inconceivable because a similar process has already been postulated for another insect-symbiont association. A newly described bacterium *Sulcia muelleri* displays “patchy” distribution across several groups of Auchenorrhyncha (Moran et al., 2005b). Considering the significant congruence between *Sulcia* and host phylogenies, this distribution has been attributed to a long-term cospeciation accompanied by many losses. However, even this impressive story is incomparable to that implied by *Buchnera*-*Wigglesworthia* common ancestry (Takiya et al., 2006). Current estimates of

insect groups' divergence indicate that such bacterium would have to maintain its symbiosis for the period of time reaching almost 400 My (Gaunt and Miles, 2002). This view is undoubtedly much less parsimonious than supposing an independent origin of several P-symbionts within a cluster of related lineages.

Coevolutionary reconstructions in symbiotic associations

Tree congruence

Coevolutionary reconstructions are among the most precarious enterprises in phylogenetics. Their main aims are twofold: (1) to identify whether phylogenetic congruence between two associated organisms is higher than expected by chance, and (2) to localize and explain the observed incongruences. One of the biggest problems in coevolutionary analysis is that incongruences can arise from fundamentally different sources: real evolutionary events (e.g., host switches and/or multiple colonizations) or methodical artifacts (incorrect topology, lineage duplication). During the several decades of theoretical work, mostly devoted to parasitological research, many algorithms were developed to disentangle these processes, each of them based on different evolutionary assumptions (Ronquist, 1995; Huelsenbeck et al., 2000; Johnson et al., 2001). In insect-symbiont research, the coevolutionary framework takes a specific form. For the clearly mutualistic P-symbionts, an entire phylogenetic congruence due to exclusively vertical transmissions is implicitly presumed. In contrast, frequent horizontal transfers seen in many "guest microorganisms" (Douglas, 1989) suggest random distribution and usually do not provoke any rigorous coevolutionary approach.

Perhaps due to these circumstances, insect-symbiont associations were only rarely addressed by rigorous coevolutionary methods. In simple cases, where both coevolving counterparts carry a strong and unequivocal phylogenetic signal, congruency can be easily determined by looking at the trees. Indeed the first confirmation of the concept of strict coevolution between insects and typical P-symbionts relied on such a visual assessment (Munson et al., 1991a; Schröder et al., 1996; Chen et al., 1999). However, many recent findings suggest that insect-symbiont associations should be seen as more dynamic systems built by multiple acquisitions, losses, replacements, and horizontal transfers. At the same time, because phylogenetic inference is far from being impervious to topological artifacts, an increasing number of taxa leads inevitably to phylogenetic inaccuracies. These, in turn, introduce false incongruence even into entirely coevolving associations.

The fact that different datasets can yield different topologies, even if produced by the same phylogeny, is common knowledge in phylogenetic theory (Hipp et al., 2004). Several cases of partial incongruences observed between insects and their symbionts during the last few years (Thao et al., 2000b; Downie and Gullan, 2005; Takiya et al., 2006; Gruwell et al., 2007) show that this danger is not only theoretical but may blur many strictly coevolving associations. In all of these studies, the authors identified significantly non-random association between insects and their symbionts, but always accompanied by several incongruent relationships. As a rule, the authors tend to explain such incongruences as being caused by a weak phylogenetic signal rather than by real biological events (e.g., horizontal transfers). This attitude should not be seen as mere prejudice: there are certainly sound reasons to take the horizontal transfers as unlikely events. The intimacy of these associations leads to the establishment of various precisely tuned adaptations that might considerably limit or entirely block any horizontal transfer. On the other hand, the degree of observed incongruence is sometimes relatively high and should not be easily dismissed

without proper analysis. For example, after finding 75% of nodes congruent between mealybugs and their P-symbionts, Downie and Gullan (2005) explained the incongruences mostly as phylogenetic artifacts, but they also acknowledged that rare horizontal transfers may have taken part in the overall pattern.

Generally, the coevolutionary approach rests on two different methodological bases, the event- and the data-based methods. The event-based analyses, such as Treefitter or TreeMap (Ronquist, 1995; Charleston and Page, 2002), try to fit two incongruent phylogenies into the “best” coevolutionary hypothesis. To do this, they optimize various evolutionary events (e.g., cospeciations, duplications, switches, and losses) on already reconstructed incongruent trees. In other words, the conflicts between the two topologies are readily accepted as real phylogenetic incongruences and the program attempts to fit the best biological explanation onto the trees. This approach is particularly reasonable in a parasitological context, where some degree of incongruence is expected as a rule (Clayton et al., 2004). In symbiosis, this kind of reconstruction has only rarely been used. For example, Takiya et al. (2006) tested the topologies of Cicadellidae and their P-symbionts with Treefitter and concluded that despite observed differences, the trees display significant overall congruency that cannot be explained by mere chance. However, in contrast to host–parasite associations, whenever coevolutionary signal is detected between insects and their symbionts, a strict congruence is the most common expectation. Thus, analysis is mainly centered on data-based methods that allow testing whether such null hypotheses can be rejected on the basis of available data, rather than the actual reconstruction of a coevolutionary scenario.

Compatibility of host and symbiont data

The question of whether two or more partitions of the dataset come from the same phylogeny is very common in phylogenetics. Incongruence length difference (ILD) (Farris et al., 1995a; 1995b) and its variants are the most frequent tests used to address such problems. Although in most cases these partitions are represented by sets of different genes collected from the same taxa, this method can be easily adopted to matrices combined from two different organisms. Due to this property, the ILD test can be applied in coevolutionary studies (Johnson et al., 2001; Lopez-Vaamonde et al., 2001; Hughes et al., 2007).

For insect symbiont associations, the ILD test was used in a few studies and allowed for both the rejection and corroboration of a null hypothesis. Results from analysis on psyllids and *Carsonella* (Thao et al., 2000b) as well as Cicadellidae vs. *Sulcia* (Takiya et al., 2006), showed that despite topological incongruences, the null hypothesis of strict cospeciation could not be rejected when the data matrices were taken as the base. A different case was reported by Downie and Gullan (2005); for a 75% topological correspondence between mealybugs and their symbiont, the overall congruence of the datasets was rejected by ILD. Considering the similarities of all of these insect–symbiont associations and the different implications of the ILD tests, knowledge of the latter’s properties and reliability becomes particularly important. There have been many debates in the phylogenetic literature on what kind of information and sources of error should be expected from this test (Yoder et al., 2001; Hipp et al., 2004; Ramirez, 2006; De Vienne et al., 2007; Quicke et al., 2007). With respect to the insect–symbiont studies, the most disquieting weakness of ILD is its sensitivity to unequally distributed homoplasies among data partitions (Dolphin et al., 2000). In a coevolutionary framework, such difference in noise content between the two partitions is very likely. Most recently, a new modification of the test, arcsine ILD, has been suggested to suppress this tendency (Quicke et al., 2007). At the time of this chapter preparation,

no study on insect–symbiont association had applied this new approach, and its power remains to be tested.

Another problem with the ILD approach is that while assessing overall congruency, it is not able to identify taxa responsible for the incongruences. A simple remedy for this shortcoming was proposed by Johnson et al. (2001). Their approach consists of removing taxa (e.g., host–parasite pairs) from the dataset until significant congruence between the partitions is achieved. This restricted set of taxa is then used to build a phylogenetic backbone common for both counterparts. Because the removed taxa are supposed to be incongruent, their position is inferred by a subsequent phylogenetic analysis constrained with the backbone topology, and the resulting trees are finally compared by a tree-based method. The efficiency of this technique to localize the source of incongruence has been recently demonstrated on a host–parasite system (Hughes et al., 2007). Due to its capacity to identify a maximal set of entirely congruent taxa, this approach might be particularly valuable for investigating insect–symbiont associations. To the best of our knowledge, it has not yet been applied to any of the known insect symbionts.

A different approach in the identification of incongruent taxa is offered by the method of partitioned Bremer support (PBS) (Baker and DeSalle, 1997). Dividing the overall Bremer support of each node to individual partitions, this method can highlight the taxa minimally contributing or even contradicting the particular node. In insect–bacteria coevolution, the partitioned Bremer has recently been used to identify possible incongruences between mealybugs and their insect hosts (Downie and Gullan, 2005). As agreed by many authors, none of the incongruence testing methods can provide definitive proof for tree congruence/incongruence and should be combined with other sources of evidence.

Tree confidence

Considering the almost inevitable errors in phylogenetic inference, the “best” tree (e.g., ML tree) is not necessarily the true tree. Thus, even if the host and symbiont trees differ from each other, the symbiont tree may not be a significantly worse explanation of the host data, and vice versa. In the realm of ML, a set of methods has traditionally been available to test the significance of tree differences with respect to the underlying data. The most popular tests are the Kishino-Hasegawa test (KH test) (Kishino and Hasegawa, 1989) and its later modifications. Although the likelihood-based tests are extremely popular and were often used, their application is not always straightforward. In the analyses of symbiotic associations, the competing topologies are trees derived directly from the data. It has been convincingly shown that under such circumstances, the KH test is not applicable (Shimodaira and Hasegawa, 1999; Goldman et al., 2000b). A modified version, the SH test developed by Shimodaira and Hasegawa (1999), overcomes this obstacle, but pays a considerable cost in the form of increased conservativeness (Goldman et al., 2000a).

For insect–symbiont coevolution, this kind of test was used, for example, by Clark et al. (2000) to assess the congruence between *Buchnera* and aphids of the genus *Uroleucon*. An important outcome of this study is the finding that an ML-based test can reject compatibility of the host and symbiont data even under high topological congruency. The author concluded that in the observed aphid-*Buchnera* system, this might be due to the heterogeneity of evolutionary process in different regions of the aphid mitochondrial DNA. Since this report, several other tests were proposed, most of them based on the ML method. Shi et al. (2005) tested several of these approaches in simulation studies and stressed the good performance of the SOWH test, a method based on parametric bootstrap (Goldman et al., 2000a). There is currently only limited experience on performance of this and other tests in

the coevolutionary field. To the best of our knowledge, the only application of the SOWH test to insect–symbiont coevolution was done in the study of Downie and Gullan (2005). Comparing phylogenies of mealybugs and their P-symbionts, the authors were able to show that the incongruence between the two topologies is not caused by sampling error.

Molecular clock and calibration of evolutionary rates

An extremely appealing method of assessing a coevolutionary scenario when topology-derived evidence is not conclusive is the comparison of speciation times. Such an approach is well known in the coevolutionary field and has even been implemented into some of its programs (Merkle and Middendorf, 2005; Charleston and Page, 2002). Ultimately, the dating of evolutionary events relies on calibration with fossil records. However, for many organisms, including parasites and symbiotic bacteria, no fossil records are usually available. The possible solution to this problem was proposed via the molecular clock concept (Avice, 1994). Where the cophylogenetic scenario is strongly supported by topological correspondence, fossil-based times of hosts can, in principle, be used to subsequently calibrate the evolutionary rate of its parasite/symbiont. For insect symbionts, this approach has been applied to several groups, such as aphids and *Buchnera* (Moran et al., 1993), cockroaches and their symbionts (Bandi et al., 1995), or psyllids and *Carsonella* (Thao et al., 2000b). The results of such calibrations revealed that the evolutionary tempo in symbiotic bacteria is considerably higher than the rates calculated for free-living bacteria (Moran et al., 1993; Moran, 1996). Such a result is not surprising as symbiotic bacteria are known to more quickly accumulate mutations due to regular and severe bottlenecks and relaxed selection.

Hypothetically, the calibrated evolutionary rates of these symbiotic bacteria could be used to determine the divergence times of other symbionts and decide between coevolutionary and multiple-origin scenarios. This method was applied to related symbionts from aphids and whiteflies (Darby et al., 2001) or anoplurans and rhynchophthirines (Hypša and Křížek, 2007). Unfortunately, despite its undeniable attractiveness, the calibration of evolutionary tempo seems to be of very limited applicability in insect–symbiont investigations. Calculations performed for various symbiotic lineages brought an unpleasantly broad range of time estimates (Table 1.2). Most important from this point of view is the considerable divergence in the evolutionary rates between two symbionts coevolving with the same host, *Sulcia* and *Baumannia* (Takiya et al., 2006).

Specificity without coevolution

An interesting phenomenon that has been largely overlooked in the insect–symbiont field is the occurrence of a false coevolutionary signal, i.e., a signal created in absence of actual cospeciation events. In such cases, the probability of a successful host switch may be constrained by phylogenetic relatedness of the hosts. As a result, the symbiont can be restricted to particular host taxa due to functional constraints rather than common evolutionary history. Empirical examples of such false congruence due to preferential switching come from the studies on primate lentiviruses (Charleston and Robertson, 2002) or host-plant specificity in psyllids (Percy et al., 2004). From a theoretical point of view, the conditions favoring false congruence were recently analyzed by De Vienne et al. (2007). They demonstrated that the higher probability of a parasite colonizing a species related to the current host can indeed lead to an extremely high degree of congruence. It would be

Table 1.2 Divergence Times Estimated for the Nodes Depicted in Figure 1.1

Node	Symbiotic Bacterium	Divergence Time (MYA)	References
1	<i>Sitophilus</i> endosymbiont	50–100	(Heddi et al., 1998)
		100	(Dale et al., 2002)
		25	(Lefevre et al., 2004)
2	<i>Riesia</i>	5,6	(Allen et al., 2007)
3	<i>Carsonella</i>	100–250	(Thao et al., 2000b)
4	<i>Portiera</i>	100–200	(Thao and Baumann, 2004b)
5	<i>Wolbachia</i>	58–67	(Werren, 1997)
		60–100	(Duron and Gavotte, 2007)
6	<i>Blattabacterium</i>	135–300	(Moran and Wernegreen, 2000)
		140–145	(Lo et al., 2003)
7	<i>Sulcia</i>	260–280	(Moran et al., 2005b)
8	<i>Tremblaya</i>	100–200	(Thao et al., 2002)
9	<i>Wigglesworthia</i>	40	(Moran and Wernegreen, 2000)
10	<i>Buchnera</i>	160–280	(Moran et al., 1993)
		100–200	(Clark et al., 2000)
		150–250	(Moran and Wernegreen, 2000)
11	<i>Blochmannia</i>	50–100	(Moran and Wernegreen, 2000)
		90–110	(Degnan et al., 2004)
12	<i>Baumannia</i>	80–175	(Moran et al., 2003)
		70–100	(Moran et al., 2005b)
		138–475	(Takiya et al., 2006)
13	<i>Nardonella</i>	100	(Lefevre et al., 2004)
14	<i>Haematopinus</i> endosymbiont	190–230	(Hypša and Křížek, 2007)

interesting to consider whether some of the insect–bacteria associations might have arisen from preferential switching instead of cospeciation processes.

Currently, there is no direct evidence of a similar host-mediated constraint affecting the distribution of bacterial symbionts. In the frequently switching bacterium of the genus *Wolbachia*, most transfers take place between unrelated hosts, and some can bridge as large phylogenetic spans as between different arthropod groups or even arthropods and nematodes (Sironi et al., 1995). However, in several cases, the monophyletic cluster of *Wolbachia* was retrieved from closely related hosts. Most recently, such a phylogenetically tight group was found in several scorpion species of the genus *Opisthophthalmus* (Baldo et al., 2007). Nevertheless, due to the lack of reliable host phylogeny, the decision between a coevolutionary and switching scenario cannot be easily made and the evolutionary interpretation remains unclear. The situation is even more conspicuous in another group of typical S-symbionts: the monophyletic lineage of *Sodalis glossinidius* (Dale and Maudlin, 1999) has been detected exclusively from *Glossina* species. At the same time, the extremely low molecular diversity among *S. glossinidius* isolates strongly indicates a recent independent origin of *S. glossin-*

idius infections in individual tsetse species. This observation led Aksoy et al. (1997b) to explain the *S. glossinidius* restriction to a narrow host group by functional constraint. If this explanation is correct, the *S. glossinidius*–*Glossina* association could provide an example of preferential switching among symbiotic bacteria.

It should also be stressed that the view of the recent establishment of *S. glossinidius* in tsetse flies rests mainly on comparison with another symbiont, *Wigglesworthia glossinidia*. This symbiont obviously underwent a long cospeciation history with tsetse flies, resulting in congruent phylogeny with *Glossina* and the considerable genetic diversification of the *Wigglesworthia* extant lineages (Chen et al., 1999). Compared to 2%–6% 16S rDNA divergence among the *Wigglesworthia* lineages, the near identity of *S. glossinidius* 16S rRNA genes seems to support its recent origin (Aksoy et al., 1997a). This view is certainly plausible and a likely explanation of *S. glossinidius* evolution. However, as noted in the previous section, the dramatic difference in evolutionary tempo observed in the two different symbionts from a single host is a warning that maximal caution should be taken when using sequence divergence as a sole criterion for dating the age of symbiosis.

The nature of *Sodalis*–*Glossina* association has been discussed in several studies with contradictory evidence. For example, the finding of practically identical isolates in various tsetse species, detection of several genetically distinct strains in a single species (Geiger et al., 2005; Geiger et al., 2007), and successful interspecific transfer of *S. glossinidius* among glossinas (Weiss et al., 2006) point toward a recent origin. In contrast, the considerable degradation of the *S. glossinidius* genome (Toh et al., 2006) is a typical sign of long-term symbiosis rather than recently established lineages. Thus, distribution and phylogeny of *S. glossinidius* cannot be unequivocally explained. Moreover, a similar pattern can be seen in yet another lineage, the bacteria of the genus *Arsenophonus* (Gherna et al., 1991). Because both *Sodalis* and *Arsenophonus* represent lineages with a rapidly increasing number of closely related symbionts reported from phylogenetically distant hosts, their investigation poses methodological problems fundamentally different from those connected to P-symbionts.

Low-level phylogeny

An attempt to reconcile phylogeny and host distribution within a consistent evolutionary framework becomes particularly difficult when the analyzed group contains both types of symbionts, coevolving mutualists, and “accidental” temporary infections. Moreover, at low phylogenetic levels, inference is further complicated by additional specific obstacles. The first is that during a short evolutionary time, the sequences gather only a limited amount of phylogenetic information. As an example, in their coevolutionary analysis on Diaspididae and *Uzinura* symbiont, Gruwell et al. (2007) reported a majority of incongruent nodes being located close to the tree terminals. They concluded that this feature is most likely due to a scarcity of information at the “shallow phylogenetic level.” Because most of the S-symbiotic associations are supposed to be of relatively young origin, their analyses are likely to suffer this shortage of information. This problem is notoriously evident in *Wolbachia*, where the exclusive usage of 16S rRNA has been largely abandoned and most analyses are now based on several rapidly evolving genes (Zhou et al., 1998; Bordenstein and Rosengaus, 2005; Casiraghi et al., 2005; Vaishampayan et al., 2007) and/or MLST-based approaches (Baldo et al., 2006; Paraskevopoulos et al., 2006). A second typical problem is that when working with closely related taxa, several genetic factors, not encountered at higher phylogenetic levels, can entirely mislead inference of phylogeny and blur a coevolutionary pattern. Various forms of polymorphism followed by incomplete lineage sorting, or intragenomic heterogeneity belong to the best known and often studied phenomena.

Intragenomic heterogeneity

In bacteria, the intragenomic heterogeneity of the 16S-23S-5S rRNA operon (rRNA operon thereafter) and its influence on phylogenetic reconstruction in closely related species has previously been shown in a taxonomically broad range of free-living species (Luz et al., 1998; Pettersson et al., 1998; Yap et al., 1999; Marchandin et al., 2003; Boucher et al., 2004; Lin et al., 2004). Moreover, it was studied in detail for the *Escherichia/Shigella* branch (Cilia et al., 1996; Garcia-Martinez et al., 1996; Anton et al., 1998, 1999; Martinez-Murcia et al., 1999), where the presence of two basic types defined according to specific tRNA gene within the intergenic spacer region (ISR) was demonstrated (Condon et al., 1995). In their comparative study of 55 bacterial spp., Coenye and Vandamme (2003) showed that the intragenomic heterogeneity can reach up to 19 nucleotide differences.

Despite all this evidence, the potential significance of this phenomenon for reconstructing coevolution in insect–symbiont associations has not yet been noted. An obvious reason for this omission is that most of the detailed coevolutionary studies have been devoted to *Buchnera*, *Wigglesworthia*, and other P-symbionts (Aksoy et al., 1997b; Chen et al., 1999; Clark et al., 2000; Sauer et al., 2000; Thao et al., 2000b). In these bacteria, the sequencing of 16S rDNA has proven to be an effective tool for deriving clear coevolutionary patterns. Thus, the intragenomic heterogeneity may not seem to pose any problems to phylogenetic analyses in symbiotic bacteria. It is, however, important to realize that the P-symbionts possess two unique features, both of which result from genome degradation and economization. First, only a single copy of the rRNA operon is present in their genomes. Second, these associations are old and the evolutionary rates considerably exceed those calculated for free-living bacteria (Moran, 1996); consequently the branches in the tree are usually long and well differentiated. In non-bacteriocyte symbionts, the situation is completely different, resembling that of free-living bacteria. For example, despite the considerable degradation of its genome, *S. glossinidius* still carries seven copies of the rRNA operon (see the complete genome Acc. No. NC_007712). For *Arsenophonus*, the complete genome is not yet available, but several lines of indirect evidence suggest that it also carries more than a single copy of the rRNA operon. First, the presence of at least two copies was reported by Thao and Baumann (2004a) in their coevolutionary study on *Arsenophonus* and whiteflies. They detected two different types of operons that were distinguishable according to tRNA genes located within the ISR. To avoid misleading phylogenetic information, they removed the ISR sequences from the alignments and used only the rRNA genes in their analysis. Based on these sequences, they reported a phylogenetically independent distribution of *Arsenophonus* bacteria within their hosts, and attributed this distribution to multiple acquisition of the symbiont. Second, related bacteria for which the whole genome is available (e.g., *Photorhabdus luminescens*; Acc. No. NC_005126) typically carry seven copies of this operon, which is consistent with many other γ -Proteobacteria.

An interesting question with strong implications for coevolutionary analyses is the degree of divergence among individual copies. This parameter determines how deep into the tree the influence of heterogeneity can reach. When individual copies of rRNA operon are extracted from the whole genomes available in the Genbank, they display a remarkable variance of intragenomic heterogeneity across the taxa. For example, very low variability is seen within *Salmonella* (Acc. No. NC_004631) or *Haemophilus* (Acc. No. NC_000907). On the other hand, in *Escherichia/Shigella*, the effect of 16S rRNA heterogeneity reaches as deep as the divergence point of the two genera (Cilia et al., 1996). This split has been estimated to have happened approximately 60 to 180 Mya (Ochman and Wilson, 1987). It is interesting to see that such a time span exceeds divergence times of many insect species and higher

taxonomic groups (Gaunt and Miles, 2002). It thus remains to be seen whether the diversity among rRNA operons in *Arsenophonus* and similar bacteria can affect our current view on their distribution in various host species.

Diversity and sample

In addition to purely methodological issues connected to phylogenetic inference, coevolutionary reconstructions are burdened by another problem: the reliability of any evolutionary interpretation depends on the sufficiency of the analyzed sample. The current pace of new symbiont descriptions being published and sequences being deposited into databases indicates that our knowledge on diversity as a whole is still incomplete. Various screenings focused on a taxonomically restricted group of insects have resulted in identification of a wealth of associated bacteria, which have been overlooked for a long time (Reed and Hafner, 2002; Weinert et al., 2007). The impact of such inadequate knowledge on overall diversity and distribution is particularly felt when analyzing frequently switching bacteria, for which coincidental synchronic detection in several unrelated host taxa is unlikely.

The problem can be illustrated by the history of *Arsenophonus* and *Sodalis* groups. Upon their description, both of these bacteria were known as unique lineages phylogenetically isolated from other symbiotic taxa, each of which was reported from a single host group (*Glossina* for *Sodalis*, *Nasonia* for *Arsenophonus*). This idea lasted until the description of additional members of both lineages (Figure 1.2B). In *Sodalis*, it took almost a decade since its phylogenetic characterization (Aksoy et al., 1995) to attain the description of the first related lineage (Lefevre et al., 2004). Moreover, the new *Sodalis* lineage turned out to be a well-established P-symbiont coevolving with its host group, the weevils of the genus *Sitophilus*. The picture became even more complex after recent descriptions of two additional lineages. First, a sister lineage to the *Sitophilus*-derived symbionts was found in a bloodsucking hippoboscids of the genus *Craterina* (Nováková and Hypša, 2007). Its phylogenetic position indicates that although tsetse flies and hippoboscids are closely related families, their *Sodalis* symbionts have been acquired independently. This arrangement is a typical illustration of sampling significance: in the absence of the *Sitophilus*-symbiont record, the symbionts of glossinids and hippoboscids would form a monophyletic lineage. This finding in turn would imply a common origin of *Sodalis* symbiosis in a pupiparan ancestor. Finally, another *Sodalis* lineage was described by Fukatsu et al. (2007) from the chewing louse, *Columbicola columbae*. The authors pointed out that similar to *Sitophilus*-associated symbionts, these bacteria are likely to display “P-symbiotic traits”: they inhabit specialized host cells, their vertical transmission includes migration to the ovary during the host ontogeny, and they are distributed worldwide together with their host species.

Even more dramatic is the history of the genus *Arsenophonus* (Figure 1.2A). This bacterium was described for the first time as a transovarially transmitted infection associated with the son-killer phenomenon in a parasitoid wasp *Nasonia vitripennis* (Huger et al., 1985; Skinner, 1985; Werren et al., 1986). Later, it was formally described as a new genus within the family Enterobacteriaceae, closely related to *Proteus* and containing a single species, *Arsenophonus nasoniae* (Gherna et al., 1991). Independent of these studies, the presence of intracellular bacteria infecting various tissues of triatomine bugs was described from microscopic studies (Louis et al., 1986; Hypša, 1993). However, only ten years later, this bacterium was phylogenetically characterized as closely related to *A. nasoniae*, and described as *A. triatominarum* (Hypša and Dale, 1997). In the absence of any other record on *Arsenophonus*, the relatedness of the two bacteria, from *Nasonia* and *Triatoma*, led the authors to discuss possible routes of *Arsenophonus* transmission between triatomines and parasitoid

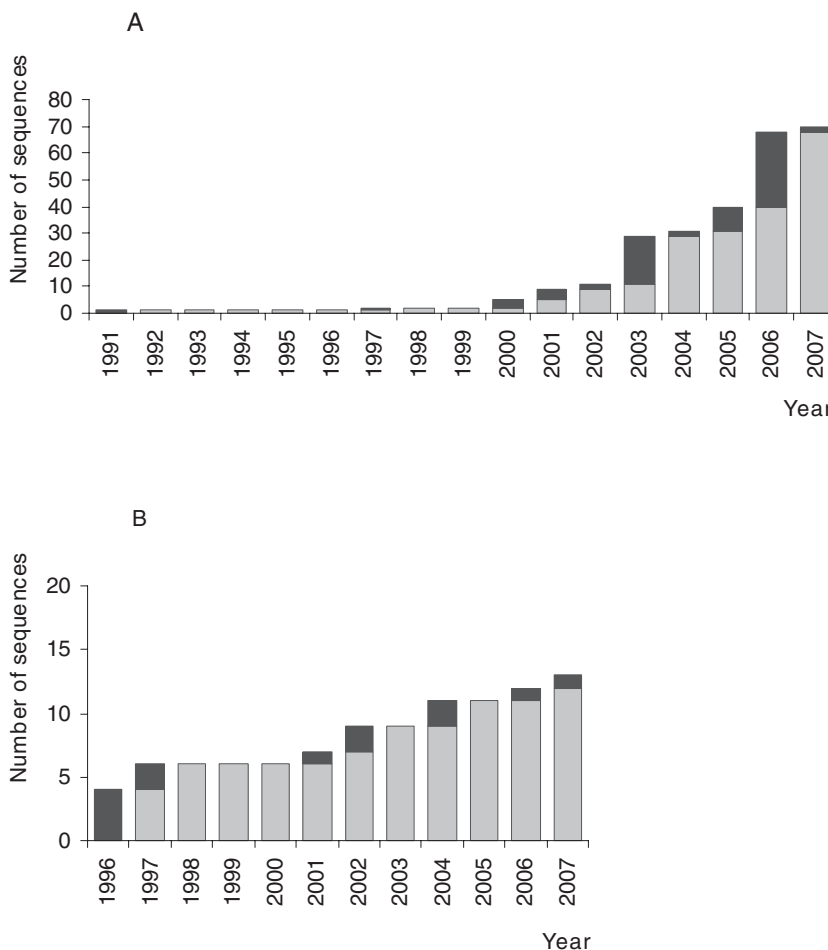


Figure 1.2 An increase of *Arsenophonus* (A) and *Sodalís* (B) records from various insect groups. The bars show cumulative numbers of sequences deposited into the Genbank; dark tops represent new records added in the given year. The sequences are deposited under the following accession numbers: *Arsenophonus*: 1991—M90801; 1997—U91786; 2000—AF263561, AF263562, AF286129; 2001—AF400474, AF400480, AF400478, AY057392; 2002—AY136168, AY136153; 2003—AY265341-AY265348, Y264663-AY264670, AY264673, AY264677; 2004—AY587141, AY587142; 2005—DQ068928, DQ314770-DQ314774, DQ314777, DQ314768, DQ115536; 2006—DQ538372-DQ538379, DQ508171-DQ508186, DQ517447, DQ508193; 2007—EU039464, EU043378; *Sodalís*: 1996—U64867-U648670; 1997—AF005235,Y11391; 2001—AF426460; 2002—AY126638, AY126639; 2004—AY72989, AY729900; 2006—EF174495; 2007—AB303382.

wasps. Since then, the number of *Arsenophonus* records is steadily increasing (Thao et al., 2000a; Trowbridge et al., 2006; Allen et al., 2007) and their current distribution makes any switching scenario meaningless.

The new findings indicate that both genera, *Sodalís* and *Arsenophonus*, may be far more abundant than what can be concluded from their currently known diversity. Moreover, they have given rise to several P-symbiotic lineages that have long been overlooked. Because

symbiont detection and identification almost invariably relies on PCR-based methods, failure to detect a particular bacterium may be a technical consequence of the incapability of the used primers on specimens from these organisms. For example, the frequently used eubacterial primers (O'Neill et al., 1992) were able to detect P-symbionts in several sucking lice, but provided repeatedly negative results with closely related species. The ubiquity of these symbionts in all of the studied species could only be proved by designing specific degenerate primers (Hypša and Křížek, 2007). The danger of mutational changes within priming sites is a notorious problem in molecular biology. It may, however, be commonplace in symbiotic bacteria, where high evolutionary rates and relaxed selection may lead to changes within otherwise conservative regions.

Conclusion

The two last decades of investigations into insect–symbiont associations can be characterized to a great extent by a shift from descriptive research to phylogenetic approach, which instigated a rapid accumulation of molecular data. Due to the unique traits of symbiotic genomes, this research often requires the development and usage of highly specific methods of evolutionary inference. They are represented by various nonstandard evolutionary models (Galtier and Gouy, 1998; Boussau and Gouy, 2006; Gowri-Shankar and Rattray, 2007), complex techniques of coevolutionary analysis (Downie and Gullan, 2005; Takiya et al., 2006), or application of various forms of molecular clock conception (Darby et al., 2001; Hypša and Křížek, 2007). It seems almost ironic that the intensive work into some areas of this research contributes more to the development of phylogenetic methodology than to the solution of actual problems. For example, after many detailed analyses, it is still not clear whether the highly reduced genomes of P-symbionts carry an unequivocal phylogenetic message or whether it has been irreversibly erased by a degradation process. In this respect, it is encouraging to note that a hope has been recently expressed by Wu et al. (2006) that newly sequenced genomes may provide “missing links” that transverse the phylogenetic gaps. They demonstrated this view by finding that when the genome of *Baumannia* is added into the P-symbiotic tree, it forms a branch shorter than usual in other P-symbionts. The focus of several recent studies indicates that the insect–symbiont field may be experiencing another methodological jump. Within the last few years, the increasing number of complete genomes available in the Genbank, and the improved efficiency of computation processes, allowed for various kinds of genome-wide comparisons (Lerat et al., 2003; Comas et al., 2007) and the search for alternative phylogenetic markers (Belda et al., 2005). At the same time, the combination of molecular methods with classical microscopy techniques made it possible to readily connect the phylogenetic data to the histological picture (Moran et al., 2005a; Moran and Dunbar, 2006; Perotti et al., 2006; Fukatsu et al., 2007). This link may be particularly important in connection to the growing number of described symbiotic lineages and the rapidly increasing complexity of symbiotic systems.

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chapter two

Self–nonself recognition in symbiotic interactions

Otto Schmidt

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Introduction

Relationships where two organisms engage in close metabolic or cellular interactions can potentially lead to mutually beneficial coexistence (Baumann, 2005). However, symbiotic coexistence is never the primary aim of these interactions, but the result of an evolutionary race, where both organisms continuously adapt to stay in the relationship (Red Queen effect). To be of mutual benefit for both organisms, both sides have to give up established pathways to engage in novel modes of action that give the new entity an overall selective advantage (Kitano and Oda, 2006). Although there is no room for compromise between the inherent virulence of the intruding organism and the defense abilities of the host insect, it is unlikely that symbiotic relationships are established if one side always wins. Only if both interacting organisms change and evolve in the process is the emergence of a truly symbiotic relationship possible. From an immunological point of view, these relationships are an evolutionary trade-off between the selective advantages gained by the two coexisting organisms versus the loss of individual integrity and independence of the host insect and the intruder. This raises the question of how we define host integrity and why insects are fighting some intruders but not others. In fact, to describe how two organisms evolve to coexist in symbiotic relationships, we have to understand how multicellular organisms such as insects recognize “self” from “nonself.” This also raises the interesting question of whether multicellular organisms are in fact the result of symbiotic relationships of genetically identical but potentially independent cells.

In the following we examine symbiotic relationships in the context of how host organisms recognize their own cells as self and other cells or potentially parasitic or pathogenic organisms as nonself. This allows us to make predictions of compatible and incompatible interactions. For example, endoparasitoid interactions, where one insect develops inside another insect, can be used as a paradigm for host manipulation and immune suppression by an intruding organism. Although complete abolition of the host defense may allow parasitoid development, it also makes the parasitized host prone to infection by other pathogens and hyperparasitoids. Thus parasitoid host interactions may provide insights into evolutionary adaptations that are necessary and sufficient for foreign cells or organisms to establish within a host organism in either parasitic or symbiotic relationships. The general concepts developed are based on observations on innate immune reactions from a diverse range of organisms, including vertebrates and invertebrates.

Self–nonself recognition

Two cells from the same organism or cells from identical twins form shared flat membranes when they interact, forming immunological (Davis and Dustin, 2004) or other synapses (Takeichi and Abe, 2005) that turn into unique-shaped cells in tissues (Hayashi and Carthew, 2004). As much as this constitutes the most visible manifestation of self-recognition that is common to all multicellular organisms (Burnet, 1971), we nevertheless have problems in explaining the process using instructive models of cell recognition (Figure 2.1). This model, which explains most cell–cell interactions, implies that driving forces shaping the membrane are exclusively cytoplasmic in origin and therefore need extracellular instructions to respond to outside influences. In the context of instructive models our perception is that cell–cell interactions in multicellular organisms are cooperative in the sense that two cells first recognize each other as “self” and then form a mutually shared flat membrane by aligning adhesive receptors (Takeichi and Abe, 2005) and cortical tension (Lecuit and Lenne, 2007) through regulation of cytoplasmic driving forces. Although

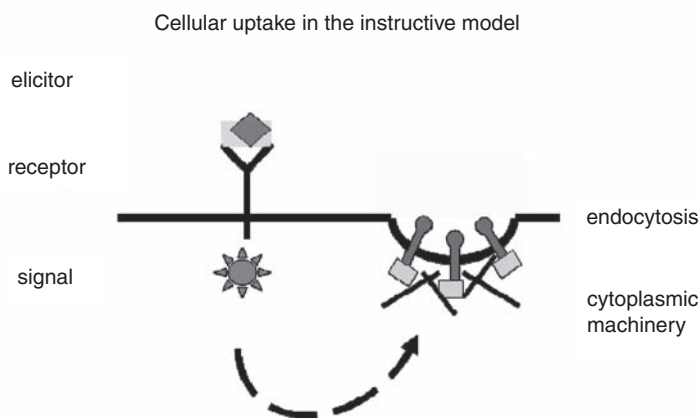


Figure 2.1 Instructive model of innate immune recognition and cellular response using receptor-mediated endocytosis as an example. Elicitors bind to receptors using “lock and key” interactions. Subsequent structural changes of the elicitor–receptor complex initiate signal pathways in the cytoplasm. In this model the signal is required to activate cytoplasmic machineries for the cell response.

instructive models can explain most cell-autonomous processes, there are some interactions that pose a conundrum.

First, there is no apparent signaling involved in self-recognition, even though the exchange of information anticipated for the formation of a flat shared membrane between two cells could be formidable, involving the fine-tuning of mutual cell surface processes, such as adhesive forces and cell turgor. Second, although there are cytoplasmic scaffolds involved in receptor anchorage (Takeichi and Abe, 2005), the driving forces shaping self-based cell interactions appear to originate from processes on the cell surface following rules of thermodynamics and energy minimization as seen in air-filled soap bubbles (Janmey and Discher, 2004). Third, self, altered-self, and nonself recognition are performed by similar gene functions, such as adhesive receptors, which is impossible to reconcile with unique receptor functions triggering instructive signaling pathways (Pradeu and Carosella, 2006). Finally, the detection of incompatibility between cells from closely related individuals of the same species is mostly due to the absence of epitopes (loss of function) that are difficult to recognize even with the most sophisticated anticipatory receptor repertoire. The fact that incompatibility is seen in “primitive” multicellular organisms, such as sponges (Fernandez-Busquets et al., 2002; Muller and Muller, 2003) or primitive chordates (De Tomaso et al., 2005), suggests that self-recognition is a process deeply embedded in the biology of cell–cell interactions comprising processes that produce multicellular organisms or tissues in the first place.

Interactive model

In the following we describe an alternative perspective involving a model that is compatible with most experimental observations on cell behavior, including the ones raised above. This interactive model describes the self-recognition process as a mechanistic process, where cells engage in adhesive interactions leading to membrane sculpturing and uptake reactions. Thus recognition involves interactions that may or may not lead to a signal, in contrast to cytoplasmic signals being a prerequisite of recognition implied in the instructive models. For example, cells that encounter each other by adhesive attachments may initially attempt to phagocytose each other. Self-recognition in this context is a defense reaction, where only cells that are identical will form a stable, shared flat membrane because that is where the mutual attempts to internalize each other come to a standstill.

The paradigm shift may be the way we look at shared flat membranes, which may not be the result of instructive signaling pathways, but the outcome of a dynamic interactive process on the cell surface involving adhesive and cellular uptake reactions that form a balance of receptor stabilization and receptor uptake reactions (Schmidt and Schreiber, 2006). In this context, self-recognition is based on cell-surface-driven processes that only require signaling when imbalances indicate altered-self or nonself.

If we accept that the outcome of interactions of cells in multicellular organisms is the result of single cell interactions that are locked in a competitive status quo among cells that would otherwise attempt to phagocytose each other, we should be able to predict cell behavior when the balance of forces is shifted, such as in tumor cells or in apoptotic cells. If self-recognition reactions are indeed based on the same mechanisms as nonself recognition, can we expect the same receptors to be involved in both reactions? More importantly, what does it take for nonself organisms to coexist inside a multicellular organism in a parasitic or symbiotic lifestyle? To answer some of these questions we have to know more about cellular uptake reactions. As mentioned before, the key to our understanding of self and nonself recognition is the potential ability of a cell to internalize an object or another

cell. The observation that some of these processes occur without signaling implies that the invagination of the cell membrane leading to phagosomes or endosomes is driven by outside forces.

Phagocytosis and endocytosis

What are these extracellular driving forces? Conceptual clues come from an almost forgotten mechanism implying a zipper-mediated phagocytosis of objects (Swanson and Baer, 1995), where surface receptors are wrapped around the object, forming a phagosome by a Velcro-like mechanism (Figure 2.2). Although Velcro-like mechanisms are visible only in few cases, such as heavily opsonized objects, it is obvious that surface properties and opsonins recruit receptors to the phagocytotic cup (Stuart et al., 2007) and given the type of opsonin, the uptake can be achieved by multifunctional receptors, including GPI-anchored receptors such as scavenger receptors (Stuart and Ezekowitz, 2005). This suggests that any particle or object potentially interacts with receptors to sculpture the cell membrane, provided it has receptor binding sites or cell adhesion proteins attached to it.

A novel conceptual insight into uptake reactions is based on the assumption that lipid particles behave like small objects. This combined with the possibility that lipid particles are sensors that change properties after encountering environmental or immunological cues (Figure 2.3) is the basis of the interactive model. As part of the recognition process,

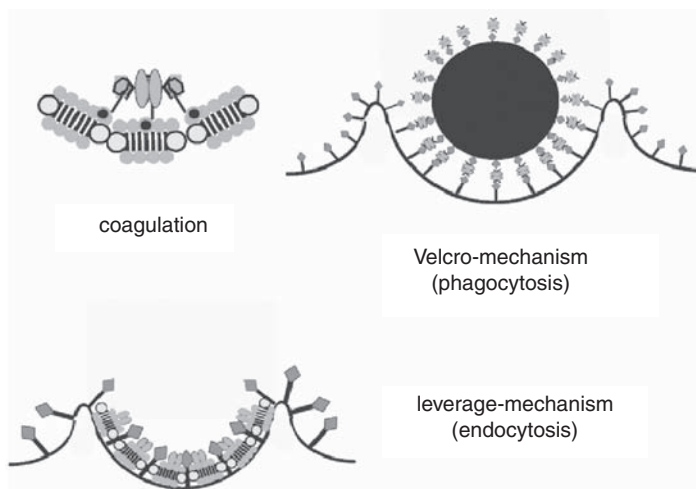


Figure 2.2 Cellular uptake of nonself objects involving attachment and interactions, leading to recognition of nonself. Adhesion and uptake of objects and microbes is driven by mechanisms that involve either Velcro mechanisms, where uptake is dependent on adhesive receptors wrapping the cell membrane around the object (black round object covered with lectins as opsonins), or cellular membrane invaginations dragging the object into the cell. Given the size differences of lipid particles and receptors, adhesive lipid particles can be regarded as opsonized objects taken up by cells using Velcro-like phagocytosis reactions. Because this involves a tilting of membrane-bound receptors around the particle, this is called a leverage-mediated (LM) uptake reaction (Schmidt and Theopold, 2004). Clustering of lipid particles on the cell surface may drive the uptake of solid and liquid cargo by a cellular clearance reaction based on dynamic adhesion processes on the cell surface (Schmidt and Schreiber, 2006).

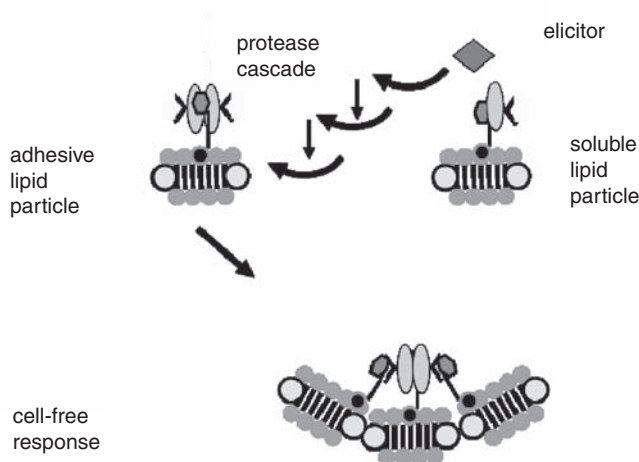


Figure 2.3 Cell-free defense reactions involving recognition and aggregation around elicitors or damaging objects. Lipid particles, which can act as circulating sensor particles, are schematically depicted as a disc of lipid bilayer surrounded by ring-shaped apolipoproteins. Associated proteins respond to elicitors (LPS) or environmental cues by becoming adhesive either directly (Mellroth et al., 2005) or indirectly through regulatory cascades (Krem and Cera, 2002). Adhesive lipid particles aggregate by cross-linking lipid particles around damaging objects or substances.

the most important change in properties is that lipid particles become adhesive (Ma et al., 2006), causing particles to aggregate (Rahman et al., 2006) or engage with cellular receptors (Figure 2.4), depending on the adhesive properties of the particle. In this context, adhesive lipid particles can be visualized as small opsonized objects that interact with opsonin-specific receptors. Although lipid uptake has been described in the literature as a classical case of receptor-mediated endocytosis reactions (Goldstein et al., 1985), this does not rule out a mechanistic process involving extracellular driving forces. In fact, the size and sensor properties of lipid particles are compatible with a possible uptake by a Velcro-like mechanism, where receptors are wrapped around the object, creating the inverse curvature of the membrane required for the uptake reaction. In an analogy with phagocytosis reactions, the interaction of adhesive receptors with lipid particles causes receptors to tilt, thereby producing a membrane curvature (Figure 2.4). Clustering of particle-receptor complexes on the cell surface will drive uptake reactions in a so-called LM-process (Schmidt and Theopold, 2004), leading to membrane sculpturing, receptor internalization, endocytosis, and phagocytosis (Figure 2.2). Thus adhesive receptors that bind to external binding sites as well as to adhesive lipid particles can potentially become involved in two opposite reactions: the cell attachment to substrate leading to cell spreading or the detachment of spread cells due to receptor internalization by LM-reactions (Figure 2.5). It is this dynamic balance of forces between two cells that only form a shared flat membrane when the two cells are identical (Figure 2.6). The unique properties of this tug-of-war between two cells is that minute alterations in the composition of these multiprotein complexes may create an imbalance, causing vesicle formation or complete phagocytosis of altered self or nonself cells and objects. Because the interactive model is based on adhesive interactions driving receptor-uptake and receptor attachment to external binding sites, the model is able to

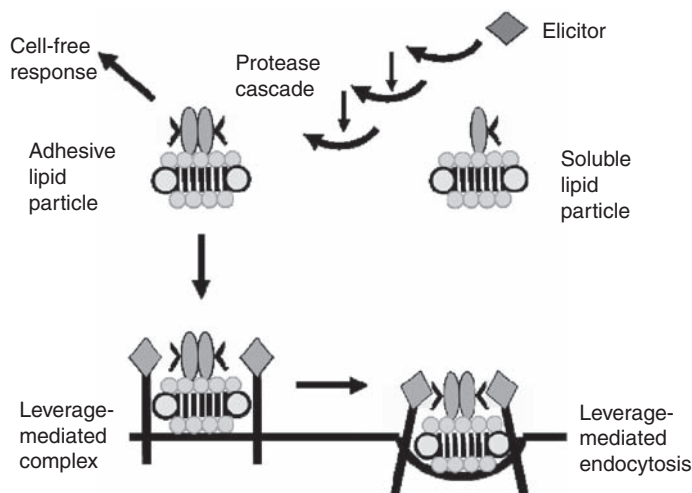


Figure 2.4 Extracellular modification of lipid particles leading to cellular clearance of adhesive lipid particles. Adhesive lipid particles can either aggregate or interact with cellular receptors, depending on adhesive binding properties. Adhesive lipid particles interact with different receptors depending on the type of lipid modification and the type of associated proteins. Lipid particles with a particular composition of nutritional lipids interact with lipid receptors on the cell surface; particles with damaged, oxidized, or nonnutritional lipids interact with scavenger receptors; and particles with microbial lipids interact with immune receptors. Each interaction can instruct cells about external cues integrating metabolic, developmental, and immune recognition functions.

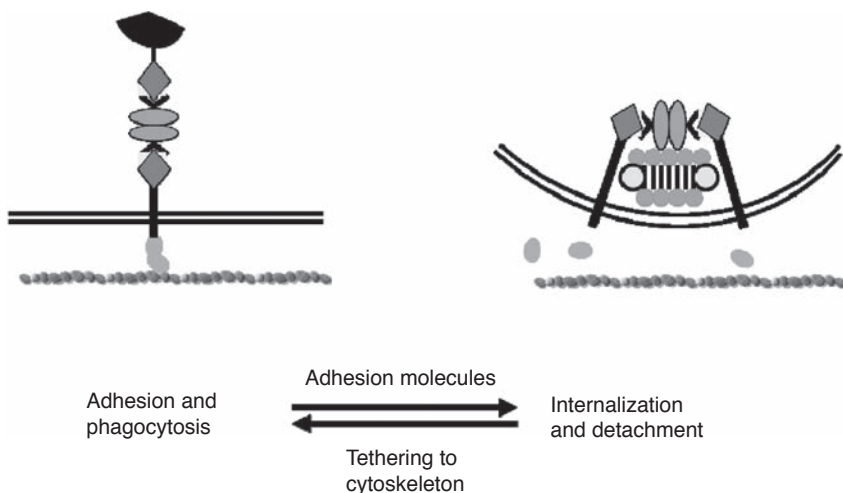


Figure 2.5 Dynamic interaction of adhesion (attachment of receptors to external binding sites) and lateral cross-linking with adhesive particles on the cell surface, causing receptor internalization (Schmidt and Schreiber, 2006). Because lateral cross-linking of receptors (receptor movements in two-dimensional membrane) are thermodynamically favored over receptor binding to external binding sites (requiring movements in three dimensions), uptake by LM-mechanisms are favored over adhesion. To retain adhesive properties on the cell surface requires anchorage to cytoplasmic scaffolds, such as actin-cytoskeleton. Conversely, destabilization of cytoplasmic scaffolds may enhance macropinocytosis of existing clusters of LM assemblies, but also prevent the formation of clusters.

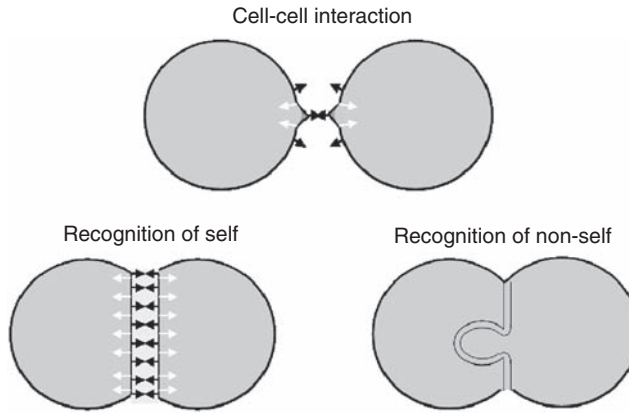


Figure 2.6 Cell adhesion and cell interaction leading to self and nonself recognition. After initial adhesive contact by adhesive receptors (black arrows) disruption of membrane attachments to cytoplasmic scaffolds allows lateral movement of receptors. This leads to leverage-mediated membrane invaginations, pulling membranes inward (white arrows) bringing the cells closer together and producing more adhesive contacts. Eventually the two cells form a shared flat surface of two adjacent membranes if the combined adhesive and LM mechanisms are equally balanced (bottom left). The implication of the model is that cells from two different genotypes produce forces at the membranes that are not equally balanced and generate vesicle formation and possible phagocytosis (bottom right).

describe seemingly opposite cell behaviors, such as cell spreading and cell detachment, as outcome of a dynamic balance between two types of adhesive receptor interactions (Figure 2.7).

Dissociation of cell contacts

Because compatible cells achieve stable interactions by forming a dynamic equilibrium between adhesive and LM-uptake reactions (Figures 2.5 and 2.6), this finely balanced symmetry between two cells is a precondition for multicellular growth and development and, consequently, is used by multicellular organisms to monitor tissue integrity. Given that interactive models describe cell–cell interactions as a tug-of-war, where neighboring cells attempt to engage in mutual uptake reactions, the question is how do individual cells detach from neighbors when they divide or acquire a new developmental fate? Unilateral destabilization of adhesive membrane proteins or reduction of LM-uptake forces is not an option, because this would lead to a reduction in surface tension and subsequent phagocytosis by the neighboring cell. In fact, the uptake of apoptotic cells by adjacent epithelial cells in nematodes (Horvitz and Reddien, 2004) could be depicted in this context. Changes in membrane composition or adhesive properties of apoptotic cells are possible factors that alter the balance of forces and drive uptake reactions, leading to phagocytosis of dead cells (Fadok and Chimini, 2001). Note that this process occurs without a need for specific signals other than a unilateral change in balance of forces between the two neighboring cells.

Thus the only way to release single cells from tissue attachments is to mutually change adhesive connections on the cell interface of all adjacent cells. One approach is for a single cell to secrete proteases into the intercellular space that cut relevant adhesion proteins, thereby simultaneously removing the attachments to neighboring cells. This may be

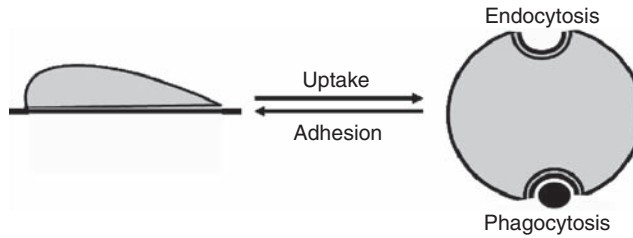


Figure 2.7 Schematic depiction of a dynamic relationship between adhesive and uptake reactions. A shift in balance toward internalization of receptors in phagocytosis or endocytosis reactions can deplete the cell surface of receptors leading to loss of adhesive abilities.

realized in Notch-regulated cell fate determination, where a single epithelial cell is detached from neighboring cells by proteolytic enzymes (Fortini, 2001). These proteases are locally restricted by lateral inhibition processes to the adjacent intercellular space, which allows the secreting cell to round up while neighboring cells retain most of their epithelial connections (Figure 2.8, dots depict proteases). Another mechanism involves the release of soluble counter-adhesion proteins, such as matricellular proteins (Greenwood and Murphy, 1998) into the intercellular space. One of the crucial properties of counter-adhesion proteins may be their ability to form strong LM-assemblies, thereby forcing the membrane-anchored adhesive receptors to internalize from the cell surface of adjacent cells against cytoplasmic receptor-anchorage (Figure 2.8, dots depict matricellular proteins). In this context, counter-adhesion proteins, such as thrombospondin, can be viewed as adhesive proteins that form LM-complexes strong enough to internalize receptors against strong cytoplasmic anchorage. The outcome of both mechanisms is the detachment of individual cells from epithelial or other tissue connections to undergo cell divisions or cell migrations.

Thus, the coexistence of different organisms can be perceived by two strategies: the adoption of cell surface properties that allow a balance of forces between the two organisms, or by a coexistence that is based on lack of cellular interactions due to detachment. While there are no clear examples of the former mechanisms in insects, certain trophic interactions in plants, such as mycorrhizal symbionts and parasitic mistletoe, may involve direct membrane alignments to exchange nutrients.

Immune suppression

Several observations suggest that immune suppressors from insect parasitoids resemble counter-adhesion proteins that drive receptor internalization in hemocytes of parasitized host insects. Although some polydnavirus (PDV) proteins are shared among different parasitoid systems (Whitfield and Asgari, 2003), the general observation is that PDV-encoded gene products identified as immune suppressors are remarkably diverse. This includes proteins with conserved cysteine patterns related to conotoxins (Cui and Webb, 1996; Summers and Dib-Hajj, 1995), cysteine-knot motif-containing proteins (Beck and Strand, 2003; Dahlman et al., 2003; Strand et al., 1997), cystatins (Espagne et al., 2004), coiled-coil containing proteins (Asgari and Schmidt, 2002; Asgari et al., 1997; Le et al., 2003), and abundantly expressed proteins without any known sequence motifs (Harwood et al., 1994; Harwood et al., 1998).

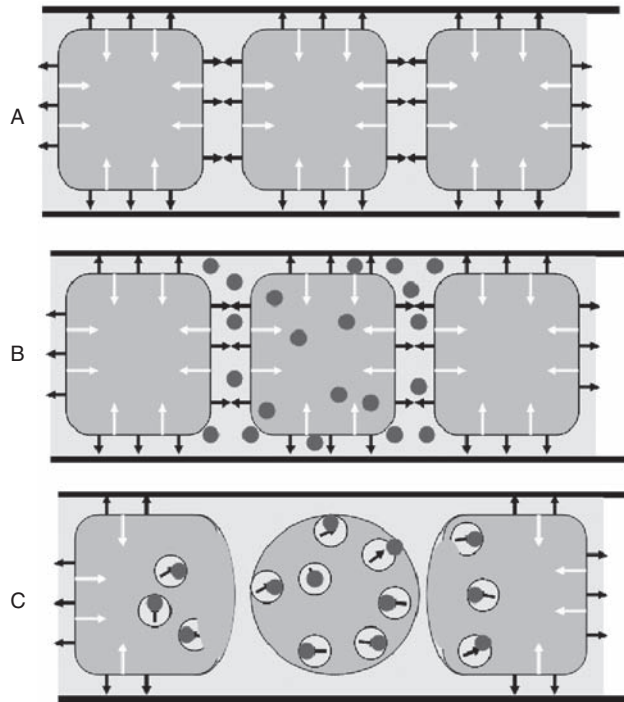


Figure 2.8 Tissue formation and cell detachment. Epithelial cells form regular honeycomb-like cell shapes by adhering to each other with adhesive receptors that may also engage in LM uptake reactions, which determines cell surface membrane tension. If one cell divides or migrates out of the tissue it has to detach from the others, which is only possible by cutting the adhesion proteins (e.g., Notch/Delta interactions are cleaved by a secreted protease) or by releasing adhesion proteins that are able to overcome the receptor stabilization provided by cytoplasmic scaffolds (e.g., matricellular proteins).

Despite the observed diversity of immune suppressors, some phenotypic commonalities are interesting to explore in terms of a possible common functional mechanism. First, most of the suppressors are secreted proteins that find their target cells after being released into the hemolymph (Cui et al., 1997). This is also true for suppressor proteins that are produced in target cells, such as PDV-infected hemocytes. Second, most immune suppressors interact with the hemocyte surface and are taken up by endocytosis or phagocytosis reactions. Finally, suppressors that target cellular immune-related functions appear to primarily affect the cytoskeleton, the most apparent being the destabilization of F-actin (Asgari et al., 1997; Strand and Pech, 1995a; Webb and Luckhart, 1994). But while suppressor proteins are internalized by target cells, the protein does not interact directly with actin in the cytoplasm. For example, expression of recombinant suppressors lacking signal peptides also lack function (Asgari and Schmidt, 2002). It appears that the suppressor causes the destabilization of F-actin in the process of being taken up by the cell.

Whether these perceived commonalities among immune suppressor functions are due to a common origin of ancestral PDV genes (Stasiak et al., 2005) or the outcome of convergent evolution (Federici and Bigot, 2003), the parasitoid factors involved in cellular inactivation of defense functions appear to target the active cytoskeleton (Shelby and Webb,

1999). The resulting destabilization mostly through actin-depolymerization appears to render hemocytes at least temporarily incapable of phagocytosis (Asgari et al., 1997) and unable to spread on foreign surfaces (Beck and Strand, 2003; Luckhart and Webb, 1996). This is associated with changes in the number of hemocytes in circulation (Strand and Pech, 1995a), and increased rates of apoptosis in cases where the suppressor persists in the hemolymph for more than a day (Beckage, 1998; Lavine and Strand, 2002; Pech and Strand, 2000; Strand and Pech, 1995b).

Because lectins resemble immune suppressors in driving uptake reactions (Figure 2.2) and in the process destabilize actin-cytoskeleton (Glatz et al., 2004) (Figure 2.5), the systemic clearance of modified lipid particles inside the hemocoel may remove adhesive receptors from the hemocyte surface and provide a model for immune suppression in general. The model implies that lectins and immune suppressors associate with lipid particles and as a consequence these modified particles are cleared from the plasma by hemocytes. Given the presence of large amounts of immune suppressor proteins in the hemolymph of parasitized hosts, facilitated by the PDV genome structure (Webb and Cui, 1998), the uptake of modified lipid particles by LM-reactions can quickly lead to a massive clearance of adhesive receptors from the hemocytes surface (Schmidt et al., 2005). This model also explains the observed gradual inactivation of hemocyte functions with increasing amounts of suppressor in the hemolymph, which implies that the functional integrity of actin-cytoskeleton is more relevant to spreading and adhesion than to pinocytosis (Glatz et al., 2004) and to some degree phagocytosis (Vilcinskas et al., 1997b). The observed dynamics of lectin-mediated cellular uptake and spreading reactions (Figure 2.7) suggest that extracellular driving forces depend on functional adhesion molecules forming a complex with lipid particles that drive cellular uptake. In contrast to lectins, suppressor-modified lipid particles in the hemolymph mediate LM-uptake reactions but no coagulation reactions. The molecular reasons for the absence of coagulation reactions in parasitized insects are not known.

It will be interesting to compare the functional properties of all known immune suppressors in this context and examine how lipid particle-mediated uptake reactions are used by each of the suppressor systems to inactivate hemocytes. For example, immune suppressors resembling conotoxins (Dib et al., 1993; Nappi et al., 2005; Parkinson et al., 2004) are similar to Kunitz-type protease inhibitors, which could bind to proteases found on lipid particles (Rahman et al., 2006) or to lipoprotein-like receptors (Kasza et al., 1997). The fact that none of the known immune suppressors binds to any of the known hemocyte receptors could indicate that interactions with lipid particles may be necessary (and sufficient) to activate adhesive proteins, such as apolipoprotein III, that interact with the hemocyte surface. In this context it is interesting to note that PDV-infected *Microplitis demolitor* hemocytes not only release a soluble suppressor but also secrete a cell-bound receptor, which is essential for de-adhesion (Beck and Strand, 2003). Although there are other explanations, the mucin-like receptor could be involved in uptake reactions similar to lectin-mediated uptake and de-adhesion reactions described above. Lectins performing different functions in immunity and tissue integrity have also been characterized in host pathogen interactions (Osta et al., 2004).

Immune avoidance and evasion

Our perception of cells responding to external cues by signaling to the cell first before mobilizing cytoplasmic driving forces (Figure 2.1) can be turned on its head by the interactive model, where cells interact with substances, objects, or other cells and in the process

may or may not generate a signal (Figure 2.4). For example, an object or a microorganism with surface binding sites that attach to any membrane-anchored receptors, including GPI-anchored receptors, is internalized by Velcro mechanisms, where the cell membrane is wrapped around the object by adhesive forces (Figure 2.2). This implies that the recognition of foreign objects and its uptake by cells is determined by the presence of adhesion protein repertoires that are capable of attaching to the surface of the intruding object or organism. In this scenario, any objects including those that have not been encountered before by the insect are taken up and internalized as long as receptors bind to it. Thus, the interactive mode of uptake can proceed with fortuitous binding activities, while the instructive mode requires specific signals as a prerequisite for cellular uptake reactions. While the two modes of interaction are not mutually exclusive, the interactive process may be a fallback mechanism when instructive modes of interactions are overcome by pathogens (Jones and Dangel, 2006). Indeed, signaling pathways may evolve as a result of repeated exposure to the same objects as part of an adaptation process that provides the insect with immediate information whether the object is food or pathogen. The observations that insects (or innate immune systems in general) can deal with synthetic substances (Lavine and Strand, 2001) has been a conundrum for recognition models in the instructive context (Matzinger, 2007), but are reduced to adhesive uptake reactions in the interactive model.

This implies that circulating cells, such as hemocytes, involved in recognizing and removing objects are expected to present a broad repertoire of diverse adhesive proteins on their surface that can interact with foreign objects. Recent observations suggest that some of these recognition proteins may be produced by alternative splicing (Dong et al., 2006; Watson, 2005) and show variability among individuals. However, if alternative splicing generates new recognition proteins by random processes, this raises the question of how self-recognition can be avoided. Another unresolved question is how these adhesive proteins are kept inactive in circulating hemocytes to avoid self-aggregation and attachment, but become activated by highly sensitive and tightly regulated activation systems that allow aggregation around damaging objects or in wound healing (Theopold et al., 2004).

This highlights the significance of extracellular regulatory cascades to recognition processes. It appears that insects, like other invertebrates with an open circulatory system, are using coagulation reactions not only for wound-healing but also for the inactivation of pathogens (Theopold et al., 2004). While these extracellular defense reactions have long escaped our notice due to the difficulty of analyzing covalently linked coagulation products at the biochemical level, it has become apparent that the regulatory cascades controlling coagulation and melanization (Cerenius and Soderhall, 2004) are part of an ancestral defense reaction that has been adapted to multiple functions in different organisms (Krem and Cera, 2002). As discussed before, lipid-containing particles, such as lipophorin (Duvic and Brehelin, 1998; Li et al., 2002) and vitellogenin (Hall et al., 1999) are known to be the procoagulants in arthropods. Together with the observations that some plasma components, including immune proteins, are associated with lipid particles (Ma et al., 2006), this provides a mechanistic basis for immune and other recognition processes (Figure 2.3), where particles change properties in the presence of environmental, developmental, and immunological cues (Schmidt et al., invited review). For example, lipophorin particles in insects interact with exchangeable lipoproteins and other plasma proteins, such as apolipoprotein III (Niere et al., 2001), prophenoloxidase and its activating proteases (Rahman et al., 2006), imaginal disc growth factors (Ma et al., 2006), and morphogens (Panakova et al., 2005). These modified particles may be involved in a range of cell-free and cellular processes,

including lipid metabolism (Canavoso et al., 2001), immunity (Whitten et al., 2004), growth and development (Panakova et al., 2005), creating a source of influences for individual cells that allows the cell to respond simultaneously. For example, lipid particles may interact with different receptors depending on the type of lipid modification and the type of associated proteins (Figure 2.4). While lipid particles with a particular composition of nutritional lipids may interact with lipid receptors on the cell surface, particles with damaged, oxidized, or nonnutritional lipids may interact with scavenger receptors. The notion that particles with microbial lipids may interact with immune receptors by activating adhesion proteins (Schmidt et al., invited review) is therefore part of a sensor function that includes and integrates metabolic, developmental, and immune recognition functions.

This raises the question of whether pathogens and parasites are less vulnerable outside the hemocoel. There are many reports that parasitoid eggs are deposited inside tissues rather than in the hemocoel to avoid encapsulation reactions (Salt, 1963; Vinson, 1990). Likewise the spread of baculoviruses from infected gut cells is assumed to occur between tracheal epithelium and the basement membrane lining the hemocoel (Engelhard et al., 1994). This begs the question of whether insects can mount a defense reaction outside the hemocoel and, if affirmative, what type of defense reaction?

Food or pathogen?

A key observation is the involvement of lipid particles as sensors and as pro-coagulant (Figure 2.4), which provides a conceptual basis for cell-free recognition and sequestration of damaging objects. These particles are able to cross basement membranes and the lining of the hemocoel to shuttle lipids (Canavoso et al., 2001) and are potentially capable of recognizing and attacking potential pathogens, such as virions, in hemocyte-free intercellular space. Moreover, lipid particles are able to move across some epithelial layers, such as follicle cells and the gut epithelium. Lipid particles in the gut lumen are involved in the extraction of lipids from food and transport to the brush border membrane of gut cells or fat body cells inside the hemocoel. Uptake of lipids from lipid droplets and food sources in the gut is mediated by lipid transfer proteins (LTPs). Because insect LTPs appear to be involved in the transfer of diglycerides (Canavoso et al., 2004), phospholipids (Golodne et al., 2001), cholesterol (Jouni et al., 2003), glycolipids (Rao et al., 2005), hydrocarbons (Schal et al., 1998), hormones (Sevala et al., 1997), and carotenoids (Tsuchida et al., 1998), the question is whether only a few proteins with broad specificities bind and transfer lipid-like molecules. Given that insect LTPs interact with human lipid particles (Sellers et al., 2003) and that human LTPs are related (Yamashita et al., 2001) and functionally similar (Levels et al., 2005) to LPS-binding proteins (LBP), this could indicate that the initial uptake of lipids from food into the lipid moiety of lipid particles is rather indiscriminate and may even include microbial lipids. Once inserted into the lipid particle, the microbial lipids may interact with recognition proteins, such as hemolin (Schmidt et al., 1993), or mediate the cross-linking of glycolipids by activated lectins (Figure 2.3), causing aggregation of particles that sequester the toxins into lipid-coated aggregates (Rahman et al., 2006). In this context, the mechanism of lipid extraction and transfer to lipid particles in the gut lumen would be associated with upstream recognition processes that allow the distinction between dietary and nondietary lipids if the latter mediate changes in particle properties, such as becoming adhesive to receptors other than lipid receptors. The more adhesive lipid particles accumulate in the gut lumen the more likely they interact with each other by cross-linking of glycolipids to form aggregates, which will sequester the LPS or other nondietary lipids and remove it by excretion. The association of immune proteins, such as

pro-phenoloxidase (Rahman et al., 2006) with lipid particles provides additional linkages between aggregation and melanization (Kanost et al., 2004).

This begs the question of whether lectin-like toxins, such as crystal toxins from *Bacillus thuringiensis* that bind to glycolipids (Griffitts et al., 2005) are inactivated by cell-free reactions in the gut lumen. If mature toxin proteins bind to lipid particles and oligomerize, the toxin may in fact become inactivated by coagulation and melanization reactions in the gut lumen before it can reach the brush border membrane of the gut epithelium (Ma et al., 2005). Because immune-induced insects secrete immune components, including lipid particles, into the gut lumen (Rahman et al., 2007), cell-free defense reactions provide a molecular concept for inducible tolerance mechanisms observed in insects against toxins (Rahman et al., 2004) and other pathogens (Sadd and Schmid-Hempel, 2006). An important question, which is not discussed here any further, is how the elevated immune status in insects is transferred from one generation to the next (Little et al., 2003; Moret and Schmid-Hempel, 2001; Rahman et al., 2004). Although genetic evidence suggests that the immune status can be transmitted by a maternal effect (Little et al., 2003; Rahman et al., 2004), the mechanism of this epigenetic transmission is far from clear (Little et al., 2005).

Immune defense and fitness costs

Insects defend themselves against intruding pathogens with an induction of a broad range of innate defense reactions (Schmid-Hempel, 2005). Among other defenses, such as synthesis of antimicrobial peptides (Hoffmann, 2003), lipid-containing particles may play important roles as immune sensors and effector particles, such as pro-coagulants (Duvic and Brehelin, 1998; Hall et al., 1999) or in detoxification (Vilcinskas et al., 1997a). This involves the modification of lipid particles in immune-induced organisms, such as changes in lipid or protein composition. One of the consequences of environmental and immune-related lipid modifications is that particles become adhesive, causing aggregation (Figure 2.3) or clearance by cells (Figure 2.4). Importantly, the modifications of lipid particles in the hemolymph are likely to reduce the capacity of the lipid carrier to transport and shuttle lipids between membranes, which is expected to impose fitness penalties in immune-induced insects. In fact, the role of lipid particles in both lipid metabolism and immunity allows for the first time the understanding of immune-related fitness costs as a trade-off between metabolic and immune functions that appear to be mutually exclusive. Although some immune proteins, such as the Toll/Spätzle receptor complex, have been known to be involved in *Drosophila* development as well as immunity (Lemaitre et al., 1996), it was assumed that the two functions were performed at different developmental stages. In contrast, coagulation reactions involving posttranslational modifications of lipid particles by environmental and immune elicitors are possible at any stage of development, reducing the pool of lipid particles available to lipid metabolism and therefore diminishing the capacity of the organism to grow. Even if modified lipid particles do not aggregate, the molecular and structural modifications may already preclude normal lipid carrier functions. Thus immune-induced insects incur delays in development and are expected to return to normal metabolic levels as soon as the exposure to environmental or immune threats is over; otherwise, immune-induced insects are outgrown by noninduced insects.

Given a trade-off between growth and immunity (Fellowes and Godfray, 2000; Kraaijeveld and Godfray, 1997; Moret and Schmid-Hempel, 2000; Schmid-Hempel and Ebert, 2003), we can now look at organismic interactions from a point of view where alternative energy-investments in different strategies may affect the overall response toward intruders. In line with this model, insects may in fact not mount an immune response when

exposed to another organism if the cost imposed of inducing an immune response is higher than the overall damage caused by the intruding organism. A corollary of these trade-offs is that insects that act as a vector of a disease to other organisms, such as mammalian animals or plants, may not respond to microbes either because the intruder has acquired immune evasion or suppression abilities or that the damage to the insect is simply less of a fitness penalty than attempts to eliminate the microbe. We have anecdotal evidence that insects with an elevated immune response are less susceptible to baculoviruses and possibly remove previously tolerated protozoan from their system after immune-induction by different elicitors (unpublished data). This suggests that the elevation of the immune status of an insect by various induction mechanisms may potentially eliminate a previously tolerated pathogen from the system and thus change the vector status of an insect. This has implications for pest management and biosecurity strategies, where some insects that carry pathogens may only be damaging to agricultural production and human health due to disease transmission. In cases where an insect is a vector of a damaging disease, the objective of eliminating the damaging organism from the insect by inducing the immune status by other means may thus be more effective than attempting to eradicate the insect.

The involvement of metabolically valuable components in immune defense provides a first conceptual framework on why immune-related fitness penalties exist and why host organisms mount a response to some microbes but not to others. It also provides new approaches to examine the role of immune defenses in the establishment of symbiotic relationships.

Intracellular defense reactions

Because lipid particles and LTPs are present in the cytoplasm (Heeren and Beisiegel, 2001) as well as outside the cell, the involvement of these particles in the sensing and inactivation of potential pathogens inside the cells should be explored. Although nothing is known about a role of LTPs inside the cell in insects, the immune-related induction of LTPs in plants (Molina and Garcia-Olmedo, 1993) and their involvement in resistance signaling (Maldonado et al., 2002) can be seen as an indication that lipid-containing particles may play a role in intracellular defense reactions.

What are the defense options for host organisms against intracellular microbes? Despite the wealth of knowledge of intracellular recognition proteins (Abramovitch et al., 2006; Hsu et al., 2007; Meylan et al., 2006; Kawai and Akira, 2006), our understanding of how intracellular microbes are inactivated in insects is still in its infancy. For example, the filarial parasite *Brugia malayi* infects muscle cells of dipteran hosts, involving the dissolution of the sarcoplasmic cytoskeletal matrix surrounding the myofibrils, causing the myofibrils and mitochondria to drift apart and lose their regular spacing. This is coupled with a reorganization of the cytoplasm near the normally developing nematode and intracellular accumulate of electron-dense material near the larva. Eventually, these form a dense layer of cytoplasm around the parasite. Fine grains of melanin-containing glycoproteins (Nayar and Knight, 1996; Nayar and Knight, 1997) begin to appear in this dense cytoplasmic layer, increasing gradually until a nearly complete capsule of melanin surrounds the larva. The time course of melanization was found to be quite variable among and even within hosts, but is increased in immune-induced host insects (Nayar and Knight, 1995). Strains of refractory or susceptible host insects were found to vary substantially in the frequency of intracellular melanization, but not in the mechanism (Chikilian et al., 1995). Although much remains to be uncovered in intracellular defense reactions, the fact that the melanized capsules resemble some of the extracellular cell-free encapsulation reac-

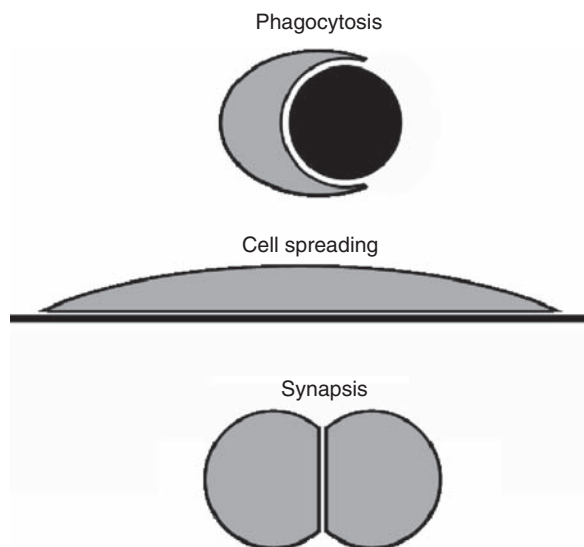


Figure 2.9 Adhesive interactions leading to phagocytosis, cell spreading, and formation of shared flat membranes between two cells.

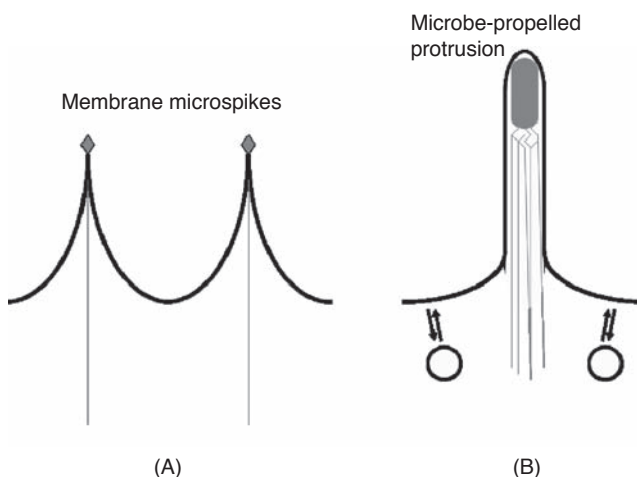


Figure 2.10 Cellular protrusions. (A) Membrane-anchored receptors attached to actin fibers form microspikes. (B) Likewise, microbes with actin fibers attached move through the cytoplasm and form membrane protrusions. Membrane protrusions are different from tip-growing cellular extensions, such as filopodia, nerve axons, and pollen tubes, where new membrane material is added at the tip, whereas in membrane protrusions new membrane is added elsewhere.

tions (Goetz et al., 1987), could indicate a similar mechanism for intracellular coagulation and melanization of microbes.

Microbes that enter cells are also able to exploit the cytoplasmic machinery to their advantage. For example, microbes move within the cytoplasm by manipulating the host polymerizing networks of actin filaments (Goldberg, 2001). Using actin-propelled movements through the cytoplasm, microbes can push membranes to form protrusions (Figure 2.10). These protrusions resemble microspikes, where membrane-bound receptors are

elevated above the cell surface by actin fibers, allowing adhesive receptors to attach to outside objects. Likewise, viruses, such as retroviruses can establish actin-stabilized membrane bridges for efficient cell-to-cell transmission (Sherer et al., 2007).

Early estimates suggest that about 10% of all insects have microorganisms living inside host cells (Ishikawa, 1989) mostly inside fat body cells or remnants of fat body cells surrounded by layers of intact cells (Smith and Douglas, 1987). Current estimates are probably much higher, given that *Wolbachia* alone infects a great variety of insect species, as well as several other arthropods and filarial nematodes, which can potentially be exploited in pest management strategies (Ioannidis and Bourtzis, 2007). Many insects feeding on plant extracts rely on a multitude of microbes to extract and digest nutrients from their food source. After evolutionary adaptation with the host (Wernegreen, 2002) different endosymbionts may coexist (Takiya et al., 2006), forming complex relationships (von Dohlen et al., 2001) and becoming genetically fixed relative to free-living species (Tamas et al., 2002). But although we know more and more about the genomic and evolutionary changes of endosymbionts, we are still ignorant of the molecular mechanisms that allow microbes to exist inside host cells. Moreover, some homopteran endosymbionts living inside somatic cells and fat body tissues found ways to exit these “hiding places” and move across the hemolymph to target ovarian cells and eventually become incorporated into the oocyte and transmitted to subsequent generations by a maternal effect (Houk and Griffiths, 1980). This trans-ovarial transmission is an intriguing process, where some of the microbes leave their intracellular location and in the process acquire additional surface coats that appear to protect the microorganism from being recognized and inactivated by host defense reactions in the hemolymph. These circulating microbes then specifically attach to ovarian calyx cells and move across the ovarian epithelium by transcytosis. While inside the calyx cell, the microbes remove some of the protective coatings to become incorporated as a symbiotic mass between the oolemma (oocyte membrane) and emerging egg shell (chorion) of the growing oocyte (Drews, 1994).

The existence of microbes inside the cytoplasm of host cells raises a number of fundamental issues, such as how immune-related protein aggregation is regulated in the cytoplasm and separate from other protein aggregates (Rajan et al., 2001). Another question is how immune-related protein aggregation is coupled with melanization reactions required for encapsulation and inactivation of extracellular (Huang et al., 2005; Shiao et al., 2001) and intracellular (Chikilian et al., 1995) pathogens. These findings have implications for innate immune responses in general, including vertebrates. For example, the tolerance to nematodes by vertebrate immune cells is mediated by symbiotic *Wolbachia* (Turner et al., 2006) via specific immune receptors (Hise et al., 2007).

Summary and conclusions

Most of our functional genomics approaches to cellular functions are based on cell-autonomous pathways and interactions, which is why the integration of metabolic, developmental, and immunological pathways in tissue and whole organisms is far less advanced than within cells. The fact that most of our cellular pathways have little to say about regulatory and energy-generating events upstream of cell-bound receptors has led to the impression that cytosolic driving forces are the only source of energy and its regulation the ultimate decision processes on how cells are shaped and membranes are sculptured. However, cellular processes exist without signaling requirements, such as self-recognition processes, involving the formation of shared flat membranes. These and other membrane sculpturing processes can be reduced to adhesive interactions resembling the Velcro-like

cellular uptake reactions of opsonized objects, where the receptors are tilted around the adhesive particles by a leverage-mediated mechanism generating a membrane curvature (Figure 2.4).

The importance of this mechanism for recognition reactions is that the cell is able to directly engage with outside objects and in the process make deductions on the possible identity of other objects or cells. This provides a new conceptual approach to recognition in general and may also provide a basis to explore symbiotic or mutually beneficial interactions.

For example, the question of self and nonself-recognition among cells can be described as a cellular defense reaction, where two cells try to engulf each other. When two cells are identical the two cells form shared flat membranes at the site of interaction (synapsis), which is the basis of tissue formation. Given the existence of genetic tools to generate chimeras, it will be interesting to revisit the conceptual question of what constitutes a multicellular organism. Because the interactive model describes cell contacts in multicellular organisms as a defense reaction resulting in a mutual coexistence between identical cells, this has implications for the integrity of organisms. One is that in individual cells that become altered genetically and/or phenotypically, the shared flat membrane generates vesicles that induce nonself signaling or the whole cell is phagocytosed by other cells. The first scenario leads to the induction of defense reactions. Melanotic tumors are one example of genetic alterations that change the cellular properties and induce defense reactions (Minakhina and Steward, 2006). The other is the uptake of apoptotic cells, which is known to occur in development and performed by macrophage-like cells (Krieser and White, 2002).

The conceptual importance of the interactive model is that the immune induction is not necessarily or exclusively based on the existence of specific recognition proteins, but also relies on a balance of forces between cells, which maintains the integrity of tissues and organisms. Any deviation of that balanced status becomes manifest as vesicle formation or other changes in cell shapes, such as phagocytosis (Figure 2.6). Other responses to changes in cell properties are the deposition of melanin (Nappi and Christensen, 2005). Interestingly, the presence of an egg shell around germ line cells may be an ancestral example of defense reactions against nonself cells emerging inside a multicellular body. Exposure of epidermal and endodermal cells to external conditions and potential pathogens produces cuticular structures and peritrophic membranes that can be traced to cross-linked aggregation and coagulation reactions. Likewise, the exposure of follicle cells to genetically different germ line cells may have resulted in cross-linked structures producing the egg chorion. Some immune proteins involved in sensing and effector functions, such as hemomucin, are indeed found on the cuticle, peritrophic membrane, and chorion in addition to hemocytes (Theopold et al., 1996).

In an evolutionary context the distinction of an insect between food and pathogens is the result of selective processes, where decisions whether to mount a response or not are at least partly based on fitness costs. The possibility that insects mount an immune response to coexisting microbes and other organisms only if overall reduction in fitness is higher than the fitness costs of an immune defense provides a conceptual basis for the fact that insects become vectors of diseases. If immune induction of an insect vector can be achieved by other elicitors, the insect may be cured from its pathogen carrier status without having to eradicate the insect. This has practical implications in pest management, when insects without the pathogen are less or not damaging.

Coexistence of microbes and other organisms that are tolerated in insects also provide a selective precondition for adaptations to new challenges involving both the insect and its passenger. For example, coexisting viruses in parasitic wasps may have become mutually

beneficial by transporting immune suppressors from the female parasitoid into the host (Pennacchio and Strand, 2006; Webb and Luckhart, 1996). In addition to understanding organismic interactions in symbiotic and pathogenic contexts, we may also have to redefine beneficial interactions of organisms to include artificial devices. New developments in transplantation technologies involving the merging of computer and biological designs will have profound impacts on our understanding of how multicellular organisms coexist with other objects.

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Is symbiosis evolution influenced by the pleiotropic role of programmed cell death in immunity and development?

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Introduction

Eukaryotes are hosts to a wide variety of microorganisms with which they intimately interact along a continuum ranging from parasitism to mutualism. Whatever the reciprocal effects of the partners, their living together creates a new living entity that emerges from this symbiosis and expresses its own extended phenotype (Dawkins, 1992). Symbiosis differs from other interspecific interactions in that the relationships between the partners are so intimate that the expression of a gene in one partner can potentially affect

the expression of any other gene in its associate, with almost unpredictable consequences on the extended phenotype. Hence, symbiosis can be seen as the integration of a foreign entity in the developmental and physiological program of an organism, both from the host's perspective and from the symbiont's perspective.

Infection usually leads to the modification of one or more host traits that may be the outcome of different scenarios: (1) modification is adaptive for the host, which responds to the infection by eliminating the symbiont in case of parasitism or integrating the symbiont in case of mutualism; (2) modification of the host trait is adaptive for the symbiont and, for example, ensures its transmission to the next generation; (3) modification of the host trait is adaptive neither for the host nor the symbiont, but rather is a correlated response to adaptive change in another trait (pleiotropy) or simply a side effect of the infection.

Pleiotropy is the ability of one gene to influence multiple traits and is a common property of many genes (Falconer and Mackay, 1996). For instance, many host genes are involved in both the immune system and development, two key determinants of eukaryote–prokaryote relationships. On one hand, any microbe infecting a host has to escape its immune system, and whenever a symbiont is detected by the host, immune genes are activated. On the other hand, a number of symbioses result in changes to host development, like the formation of root nodules in legumes, bacteriomes in insects, or galls in plants attacked by insects.

In this chapter, we propose the hypothesis that mechanisms to evade the host defense system may predispose the integration of symbionts into the host developmental program. This idea was initiated by the recent discovery that removing the bacterium *Wolbachia* from the hymenoptera *Asobara tabida* triggers programmed cell death (PCD) in egg chambers, rendering females incapable of producing eggs (Pannebakker et al., 2007). PCD is a central feature of development and stress reactions, including infection of multicellular organisms. After a brief general overview of PCD, we discuss its role in immunity. We then analyze the case of *A. tabida* related to the role of PCD in insect oogenesis. Using examples from other symbioses (sometimes far from insect symbiosis), we then develop an evolutionary perspective on how the pleiotropy of PCD in both the immune response and the developmental programs offers great potential for the evolution of symbiosis.

A brief overview of programmed cell death

In multicellular organisms, three types of cell death are currently distinguished. The first two processes, apoptosis and autophagy, are genetically controlled cellular processes that are defined by distinct morphological features and are grouped under the more general term of PCD. PCD contrasts to the necrotic cellular death that is so far considered to be a nonprogrammed process.

Necrotic cells are characterized by cytoplasmic features such as the rupture of the plasma membrane and the swelling of cytoplasmic organelles, including mitochondria. All these phenomena induce an inflammatory response. In clear contrast, the plasma membrane of apoptotic cells remains intact and organelles appear morphologically normal. In addition, apoptosis triggers the condensation and fragmentation of DNA and the blebbing of the nuclear envelope. Dying cells eventually form vacuoles that are phagocyted by adjacent cells. At the molecular level, the hallmark of apoptosis is the sequential activation of cysteine proteases called caspases, the direct effectors of the apoptotic program.

Molecular mechanisms of apoptosis occurring in normal host development and homeostasis involve pro-apoptotic and anti-apoptotic molecules (reviewed in Vaux et al., 1994). In the extrinsic pathway, initiation of apoptosis occurs through stimulation by the cog-

nate ligands (Fas, DD) of surface death receptors that belong to the tumor necrosis factor receptor (TNFR) family. This activates proteases (caspases) that may destroy DNA-repair enzymes and allow nucleases to degrade DNA in apoptotic cells. The intrinsic apoptotic pathway is initiated by intracellular signals of stress-inducing stimuli, such as reactive oxygen species, that destabilize the integrity of mitochondrial membrane, allowing internalization of pro-apoptotic proteins of the Bax family. These proteins belong to the larger Bcl-2 family that also includes the antiapoptotic Bcl-2 and Bcl-X_L proteins (Reed, 1994). Their internalization results in release of cytochrome c and the formation of apoptosome, thus activating caspases. In both the extrinsic and intrinsic apoptotic pathways, caspases are crucial and they are regulated by members of the inhibitor of apoptosis (IAP) family proteins (Miller, 1999).

Autophagy is considered as the alternative type of PCD. This process allows the degradation of intracellular components that are trapped into vacuoles and degraded by the lysosomes. Largely ignored for decades, autophagy has recently regained considerable interest, notably because of its role in promoting the survival of cancer cells (Mathew et al., 2007). In contrast to apoptotic cells, autophagic cells are defined by cytoplasmic criteria, such as the accumulation of autophagic vacuoles (Lockshin and Zakeri, 2004). Although autophagy does not seem to rely on caspases, both PCD processes act synergistically in many tissues.

Since its discovery, PCD has been found to play a central role in homeostasis and development of all multicellular organisms investigated so far, including insects, nematodes, and mammals (Jacobson et al., 1997), and in defense against tumoral transformation or infection (Williams, 1994). However, it is important to distinguish cell death triggered by external or accidental causes (infections, tumorigenesis) from cell death as a proper developmental mechanism.

PCD-mediated defense and counterdefense in host–pathogen interactions

Most eukaryotes that face pathogenic microbes have developed a set of mechanisms to confine and clear up infectious agents. These include phagocytosis, the secretion of harmful compounds such as cytokines, antimicrobial peptides, and reactive oxygen species. However, the observed rapid induction of PCD (apoptosis in most cases) in response to microbe entry suggests that PCD may constitute a basic front-line defense mechanism to arrest infection by pathogens (Williams, 1994). Conversely, increasing evidence indicates that many microbes have evolved various mechanisms to escape or manipulate host cell apoptosis to benefit their intracellular survival and proliferation (Weinrauch and Zychlinsky, 1999). Both host- and pathogen-mediated modulation of apoptosis reinforces its key role, alone or in combination with other mechanisms, in determining the evolution of host–pathogen interactions (Figure 3.1).

PCD as host defense mechanism to infectious agents

The pioneer molecular evidence that apoptosis can prevent pathogen dissemination was brought by experimental studies of adenoviruses, as infection of human KB cells by an EIB 19 kDa-deficient viral strain resulted in Bcl-2 protein expression, leading to an enhanced cytopathic effect and DNA degradation of both the infected cells and the virus (Thomson, 2001). As the corresponding wild-type strain did not induce early cell death, this also

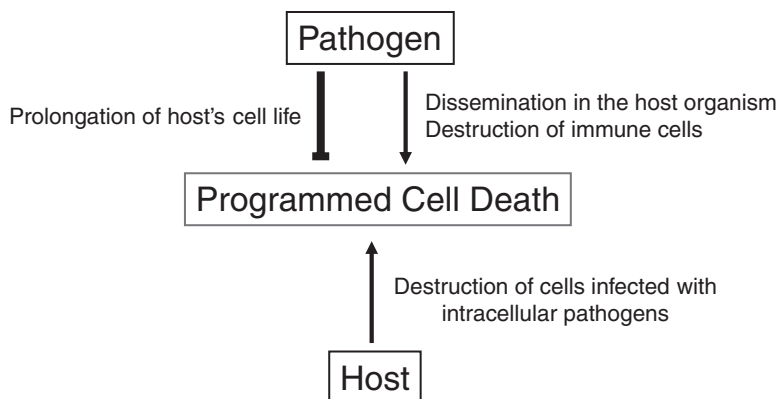


Figure 3.1 A schematic representation of the role of PCD in host–pathogen interactions. The host senses the invading pathogen and induces PCD (apoptosis or autophagy) of infected cells, destroying concomitantly the infectious agent. Conversely, to successfully infect the host the pathogen subverts its immune system by inhibition or activation of PCD, allowing its persistence and spread.

suggested the potential of the virus to regulate apoptosis. In vertebrate cells, the prevention of infection through induction of apoptosis has also been shown in response to other viruses, including encephalomyocarditis virus and influenza virus (Fesq et al., 1994; Yeung et al., 1999). Such infected cells produced a double stranded RNA-dependent kinase (PKR) and RNase L, two mediators of apoptosis (A. Zhou et al., 1998). Interestingly, recent studies have shown that induction of apoptosis serves as innate immune defense to inhibit cross-species infections of Cytomegaloviruses from rodents to human cells (Jurak and Brune, 2006). In insects, evidence that apoptosis is involved in antimicrobial defense mechanisms was first demonstrated experimentally on the *Autographa californica* M-nucleopolyhedrovirus (AcMNPV), a member of the baculoviruses. Infection of cell lines or larvae of *Spodoptera frugiperda* with a mutant strain of AcMNPV bearing inactivated *p35* apoptosis suppressor gene resulted in drastically reduced viral replication and infectivity (Clem et al., 1994). Subsequent studies showed a widespread apoptosis in *S. frugiperda* larvae injected with the mutant compared to wild-type strain (Clarke and Clem, 2003).

Since these initial studies on viruses, host cell apoptosis was found to be involved in arresting infection of many other infectious agents, including protozoan, bacterial, and fungal pathogens (Vaux et al., 1994; Williams, 1994). For example, apoptosis-like death in mosquito cells infected by malaria parasites of the genus *Plasmodium* is associated with caspase-like activity (Hurd et al., 2006). *Shigella* or *Mycobacterium* infected macrophages undergo apoptosis to reduce or stop infection process (Zychlinsky and Sansonetti, 1997). Initiation of apoptosis is based on the detection of molecular structures that are unique to the group of such microbes: the so-called “pathogen-associated molecule patterns” (PAMPs), also more generally referred to as “microbe-associated molecule patterns” (MAMPs) by some authors (Koropatnick et al., 2004), are represented mainly by components of the cell wall such as lipopolysaccharide (LPS), peptidoglycan (PGN), lipoteichoic acids, and glucans. Detection of PAMPs by the host is triggered by “pattern recognition receptors” (PRR) in the host. Although the best known PRRs, Toll-like receptors (TLRs), are described to elicit mainly antimicrobial and inflammatory responses, recent reports suggest that they may also induce apoptosis in response to microbial infection (reviewed

in Salaun et al., 2007). For instance, bacterial LPS-triggered apoptosis in endothelial cells was shown to be linked to TLR4 pathways (Bannerman and Goldblum, 1997). In mammal cell lines and in mice, TLR4-mediated apoptosis confers resistance to pneumococcal infection upon recognition of virulence factor pneumolysin (Srivastava et al., 2005). Another protein family named NLR/NOD/CATERPILLER/CLR family has been demonstrated to play a role in host innate immune responses to invasive bacteria (Ting and Davis, 2005). For example, NOD proteins were shown to be able to recognize specific glycopeptides derived from bacterial PGN (Royet and Reichhart, 2003), resulting in the activation of apoptosis through nuclear factor kappaB (NF- κ B) (Girardin et al., 2003) or caspase-8-dependent pathways (da Silva Correia et al., 2007), allowing elimination of invasive pathogens.

Modulation of host PCD by intracellular pathogens

In the evolutionary process of host-pathogen confrontation, infectious agents have evolved various mechanisms that inhibit apoptosis in the host cells to facilitate their replication and persistence, as well as mechanisms that conversely induce apoptosis to destroy immune host cells to aid their escape. Microorganisms able to modulate their host apoptosis include free-living and obligate intracellular microbes belonging to a wide range of taxa (Roulston et al., 1999; Gao and Kwaid, 2000). Here, we will discuss the molecular mechanisms employed by intracellular pathogens to modulate apoptosis in two opposing ways, e.g., inhibition or induction, and the relationship between this apoptosis modulation and the invasive processes.

Apoptosis inhibition and host infection

Apoptosis inhibition by baculoviruses is the hallmark of pathogen-modulated apoptosis leading to host infection. These viruses were first found to block apoptosis during infection of their hosts by expressing anti-apoptotic proteins such as P35 and its relatives, as well as IAP-like proteins, thereby allowing viral replication and dissemination (reviewed in Clarke and Clem, 2003). Synthesized both during early and late infection, the 35 KDa product of *p35* apoptosis suppressor gene contains a caspase cleavage site that recognizes a wide range of effector caspases (Q. Zhou et al., 1998). Consequently, P35 is able to inhibit caspases from many organisms, including those from mammals and invertebrates. Several other viral proteins with antiapoptotic activities include Op-IAP from *Orgyia pseudotsugata* nuclear polyhedrosis virus, FLICE inhibitory proteins from herpes viruses, and CrmA protein from Cowpox virus (Ploegh, 1998; Shen and Shenk, 1995; Shi, 2002). To achieve persistent infection, some pathogenic viruses use various anti-apoptotic strategies; Poliovirus and HIV are able to reduce the level of the PKR apoptotic mediators, whereas African wine fever virus and herpes virus subvert the host defense by producing apoptosis inhibitory Bcl-2 analogs that block mitochondrial membrane permeabilization, resulting in inhibition of the host apoptotic response (Hasnain et al., 2003).

In addition to viruses, pathogenic bacteria are also able to inactivate apoptosis by modulating both extrinsic and intrinsic pathways. The facultative intracellular bacterium *Mycobacterium tuberculosis* is able to inhibit apoptosis of macrophages in which it multiplies and persists. To that end, *M. tuberculosis* enhances production of TNF receptors, which in turn activate NF- κ B pro-survival signaling pathway (Balcewicz-Sablinska et al., 1998). Another facultative intracellular bacterium, *Bartonella henselae*, is able to inhibit apoptosis of endothelial cells by suppressing caspase activation and DNA fragmentation (Kirby and Nekorchuk, 2002). Obligate intracellular bacteria of the genus *Chlamydia* have been shown to protect infected cells from apoptosis during early phase of infection by blocking defense

pro-apoptotic caspases or releasing of cytochrome c from mitochondria (Fan et al., 1998). Similarly, strict intracellular pathogenic bacteria belonging to the order Rickettsiales possess the ability to inhibit apoptosis. For instance, *Rickettsia rickettsii* can suppress apoptosis in the host-infected cell via activation of NF- κ B pathway (Clifton et al., 1998), whereas *Anaplasma phagocytophilum* inhibits apoptosis of infected neutrophils through transcriptional up-regulation of the antiapoptotic gene *bfl1* and inhibition of mitochondria-mediated activation of caspase 3 (Ge et al., 2005).

Finally, many intracellular protozoan parasites such as *Leishmania*, *Plasmodium*, *Toxoplasma*, and *Trypanosoma* are also reported to possess antiapoptotic capacities that allow them to invade mammal hosts by triggering various known apoptosis cell checkpoints, including Bcl-2 and NF- κ B pathways (reviewed in Heussler et al., 2001).

Apoptosis activation and host infection

Activation of host cell apoptosis often benefits the microbes in their attack of the host and in their entry into or escape from the targeted tissues. As is the case for apoptosis inhibition, many reports show that viruses use both caspases- and mitochondrion-dependent pathways of apoptosis activation. This capacity to specifically induce apoptosis confers advantages to viruses for their replication and persistent transmission among hosts (reviewed in Thomson, 2001).

During the infection process of *Mycobacteria* into macrophages, apoptosis is induced by the binding of mycobacterial cell wall to TLR2 with subsequent activation of pro-apoptotic TNF and caspase-1 pathways (Rojas et al., 1999). Interesting cases of bacterium-induced apoptosis are found in the intracellular pathogens *Shigella* and *Salmonella*. To escape from macrophage environments, these bacteria translocate their effectors through a type III secretion system (TTSS) into the cytosol. Shiga toxin (IpaB) and its *Salmonella* homolog (SipB) bind and activate caspase-1, resulting in macrophage apoptosis (Hilbi et al., 1998; Hersh et al., 1999). Similarly, *Yersinia*-induced macrophage apoptosis requires secretion of YopP and YopJ effectors that are exported through a functional TTSS (Mills et al., 1997), and this has been shown to facilitate systemic infection of mice (Monack et al., 1998). During the late phase of macrophage infection, the obligate intracellular bacterium *Coxiella burnetii* stimulates the production of TNF-mediated apoptosis associated with a release of IL-1, suggesting also the involvement of the caspase-1 pathway (Dellacasagrande et al., 1999).

Dual inhibition and activation of apoptotic activities and host infection

Many pathogens can employ both pro-apoptotic and anti-apoptotic activities to promote their replication and dissemination. This apparently paradoxical behavior is found necessary for the intracellular survival or persistent infection of many viruses and some bacteria such as *Chlamydia* and *Mycobacteria* (Aliprantis et al., 1999; Miyairi and Byrne, 2006). It is suggested that, at the early stage of infection, antiapoptotic activity may allow for replication and generation of a critical number of infectious entities in the primary targeted host cells. Then, subsequent activation of pro-apoptotics at later stages facilitates the proliferation of infectious agents to the other surrounding host cells. Cell tropism is also considered to act in dual pro-apoptosis and anti-apoptosis exhibited by a particular pathogen species such as *Chlamydia*: active inhibition of apoptosis occurs first in cells such as phagocytes in which the infectious agents multiply, whereas early active induction of apoptosis can be seen in other cells that mediate the immunity response such as T-cells (Miyairi and Byrne, 2006). It is interesting to point out that the complex interactions that occur during the modulation of apoptosis often involve many host gene products. However, in some cases only a single pathogen gene product is required. First shown in baculoviruses (Clarke and Clem, 2003), recent reports have demonstrated the implication of a single gene determi-

nant in pro-apoptotic or anti-apoptotic activities in many other microbial pathogens. For instance, in *Photorhabdus*, a bacterium with alternate pathogenic and symbiotic behaviors, it has been shown that one gene named *mcf*, when expressed into *Escherichia coli* allows the transconjugant to persist and induce cell apoptosis, thus killing infected insects (Daborn et al., 2002). In *M. tuberculosis*, a single gene *nuoG* was found also sufficient to induce inhibition of host cell apoptosis (Velmurugan et al., 2007).

PCD, development, and symbiosis: the case of Asobara tabida

In addition to pathogens, numerous multicellular organisms form permanent associations with other microorganisms. Insects are, for example, prone to infection with intracellular maternally transmitted bacteria. Very little is known about how these bacteria evade the host immune response and the role of immunity in the control and expression of the symbiosis. Recent advances, however, suggest that pathogenic, commensal, and mutualistic microorganisms share common strategies for sustaining infection (e.g., Goebel and Gross, 2001; Dale et al., 2002; Heddi et al., 2005; Anselme et al., 2006; Silver et al., 2007).

These permanent associations, however, provide extraordinary manifestations of how bacteria can shape the development of their hosts. Two main general strategies can allow these symbionts to be maintained in host populations (reviewed in Werren and O'Neill, 1997). First, the symbionts can bring an advantage to the host. This is the case for primary obligate symbionts in insects, which provide their hosts with nutrients that are lacking from their diet (reviewed in Baumann, 2005). Without their symbionts, the hosts are unable to develop or reproduce normally. The most striking change induced by these symbioses is the development of specialized organs that are devoted to the hosting of the symbionts. However, very little is known about the evolutionary and anatomical origins of these cells (but see Braendle et al., 2003). As a second strategy, symbionts may manipulate their hosts' reproduction in a number of ways to increase their own transmission (reviewed in Werren and O'Neill, 1997). Some of these reproductive manipulations are clearly linked to modifications of the host development, like the feminization of genetic males in isopods where *Wolbachia* blocks the development of the androgenic gland (see Chapter 12) or the induction of male killing leading to an arrest of development of infected males (reviewed in Hurst et al., 2003). Recent characterization of the phenotype of male death caused by *Spiroplasma* in *Drosophila melanogaster* demonstrated that the timing of male death is strictly controlled and that PCD is highly expressed (Bentley et al., 2007). It is not clear, however, whether the relation between male embryonic death and PCD is causal. Another striking example is found in the hymenoptera *Asobara tabida* where *Wolbachia* has been found to be obligate for host oogenesis (Dedeine et al., 2001), showing how a symbiont can rapidly take control of a developmental feature of the host.

A. tabida is infected with three *Wolbachia* strains, but only the strain named *wAtab3*, is involved in the control of host oogenesis while the two other strains induce cytoplasmic incompatibility, the most common reproductive manipulation induced by *Wolbachia* (Dedeine et al., 2004). As soon as *wAtab3* is removed from the host, females are sterile. Although European populations are completely dependent on this *Wolbachia* strain to produce eggs, American populations are able to mature some eggs in absence of the symbiont. However, these eggs are smaller, and although they hatch, larvae show delayed development and die before metamorphosis (Dedeine et al., 2005). Hence dependence remains complete, but the ovarian phenotype is variable. Backcrosses of European and American populations have shown that this variability is under the unique control of the host genotype. Cytological analyses using DAPI, Acridine Orange, and TUNEL apoptosis-

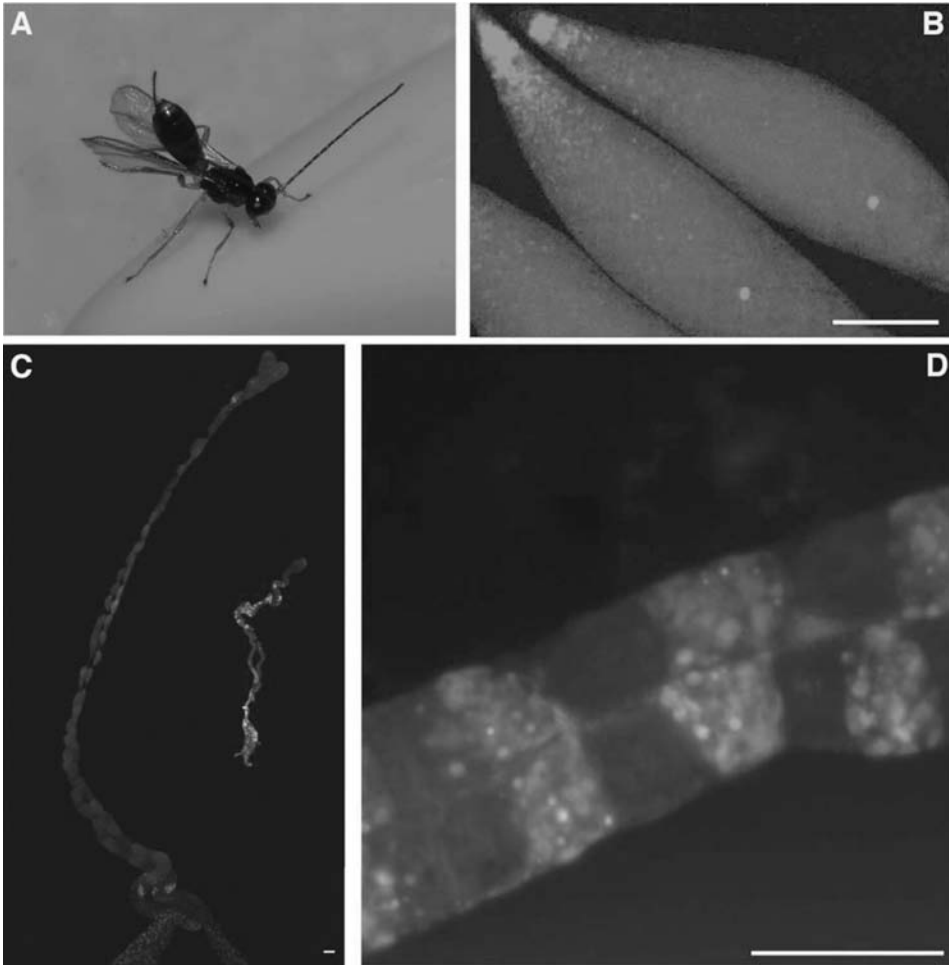


Figure 3.2 (Color figure follows p. 238.) The role of PCD in the interaction between *Wolbachia* and *Asobara tabida*. (A) An *A. tabida* female. (Picture from FV and F. Debias.) (B) Oocytes of *A. tabida* infected with *Wolbachia*. Bacteria appear clustered at the posterior end of the oocyte (at the top of the picture). Scale bar = 50 μm. (Picture adapted from Dedeine et al., PNAS, 2001.) (C) Ovaries from infected (left) and uninfected (right) females. Note the difference in the size of the organ when the symbiont is removed. Scale bar = 50 μm. (Picture adapted from Pannebakker et al., PNAS, 2007.) (D) Part of the ovary of an uninfected *A. tabida* female stained with TUNEL. Staining appears specific to the nurse cells of the egg chamber while no PCD is detected within the oocytes. Scale bar = 50 μm. (Picture from BAP.)

specific staining have revealed that in absence of *Wolbachia*, extensive PCD occurs during oogenesis in both the American and European populations (Figure 3.2, Pannebakker et al., 2007, BAP unpub. results). Before we discuss the *A. tabida* case in further detail, it is useful to briefly review what is known about the involvement of PCD in insect oogenesis.

Insect oogenesis

Most of our knowledge of insect oogenesis is based on the *Drosophila* model (for review see King, 1970; Spradling, 1993), which exhibits a pattern that can be applied to many other

insects species, including *A. tabida*. In insects, ovarian follicles are called egg chambers. Each chamber constitutes a defined number of germ line cells surrounded by a layer of somatic cells called follicle cells. Egg chambers are produced in the germarium, the anterior end of each ovariole that contains the stem cells. Egg chambers then proceed down the ovariole as they progress through 14 defined stages (King, 1970). In the germarium, the asymmetric divisions of germ line stem cells generate cystoblasts that undergo four (*Drosophila* and *Asobara*) or five incomplete divisions to form a cyst of 16 or 32 germ cells that remain connected with cytoplasmic bridges called ring canals. A single cell per chamber differentiates into an oocyte and enters meiosis. The other 15 or 31 cells become polyploid nurse cells that produce all the RNAs, proteins, and organelles required for the growth and maturation of the oocyte. These products are transported from nurse cells to the oocyte through the ring canals. By stage 8 of oogenesis, oocytes begin to increase in volume during the process of vitellogenesis, the synthesis and accumulation of yolk proteins. At the end of oogenesis, nurse cells enter PCD and expel their cytoplasmic content through the ring canals to the oocyte, a process called dumping. Follicle cells that surround the full-grown oocyte secrete proteins that constitute the vitelline membrane and the chorion.

PCD during insect oogenesis

In the complex process of oogenesis, cell death occurs at three distinct stages (Buszczak and Cooley, 2000; McCall, 2004). Two of these processes are checkpoint mechanisms that regulate the assembly line of egg chambers in response to intrinsic or environmental cues. The third one is a developmental PCD that selectively eliminates nurse cells and follicle cells at the end of oogenesis. Recent research in *Drosophila* has revealed that these cell death mechanisms rely on noncanonical genetic pathways, making insect oogenesis a particularly interesting model for exploring PCD (Figure 3.3).

The germarium checkpoint

Under poor nutritional conditions, *Drosophila* oogenesis is considerably reduced and ovaries appear small due to the lack of mature oocytes. This adjustment of oogenesis in unfavorable environmental conditions is a direct consequence of slower proliferation rates of both germ line and somatic stem cells (Drummond-Barbosa and Spradling, 2001). In addition, nutrient limitation is associated with a high incidence of apoptosis in the cysts of region 2a/2b of the germarium. It has been proposed that germarium PCD could be triggered in response to an imbalance in somatic and germ cell numbers: a limiting number of follicle cells would result in the elimination of abnormal cysts by apoptosis (Drummond-Barbosa and Spradling, 2001). The germarium checkpoint has been shown to be under the genetic control of the *daughterless (da)* gene, which encodes a Class 1 helix-loop-helix (HLH) transcription factor (Smith et al., 2002).

PCD at mid-oogenesis

Another important checkpoint occurs at mid-oogenesis before the onset of vitellogenesis, i.e., the production of yolk proteins and their storage in the rapidly growing oocyte. This mid-oogenesis checkpoint affects stage 7/8 egg chambers, when the oocyte is already specified and is positioned at the posterior extremity of the follicle. Degeneration of mid-oogenesis egg chambers can be activated in response to limited nutrients. However, abnormal or damaged egg chambers are also under the control of this checkpoint, suggesting that it allows a quality control of follicles before the costly process of vitellogenesis. Despite the diversity of signals that trigger the mid-oogenesis checkpoint, dying egg chambers

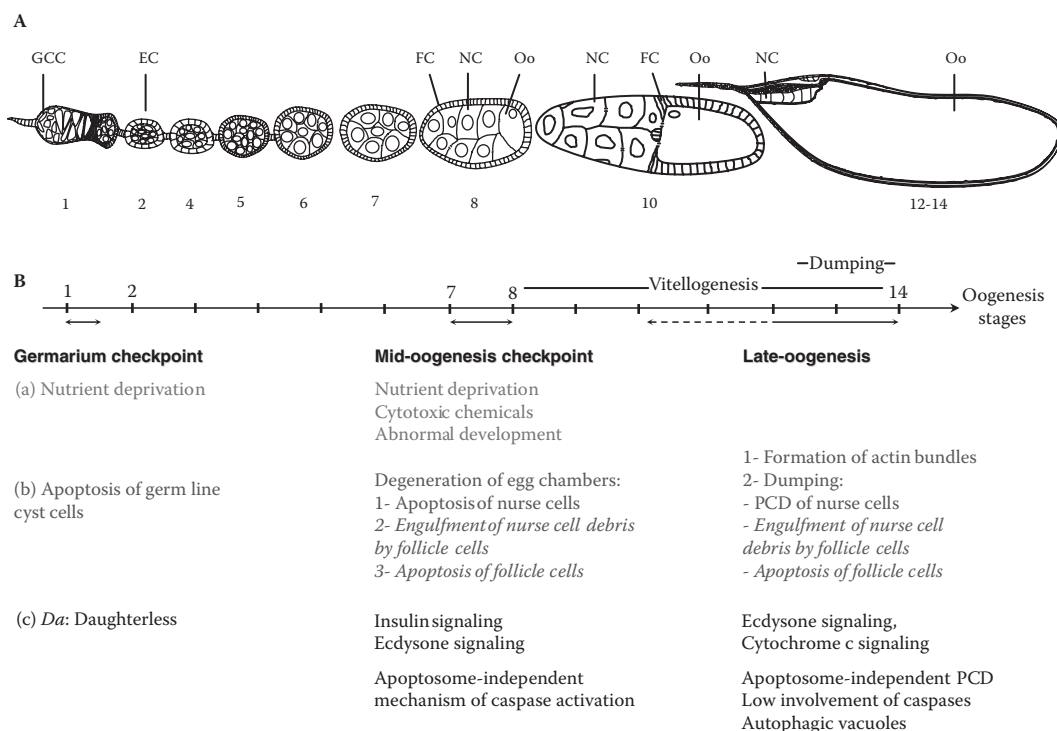


Figure 3.3 PCD during *Drosophila* oogenesis. (A) Schematic representation of ovariole structure. Egg chambers are produced in the germarium and proceed down the ovariole as they progress through 14 defined stages. GCC: germ line cyst cell, EC: egg chamber, FC: follicle cell, NC: nurse cell, Oo: oocyte. (B) Main features of PCD during *Drosophila* oogenesis. Three different stages exhibit PCD events whose characteristics are defined by: (a) conditions inducing checkpoint, (b) cytological changes, and (c) genetic pathways involved in PCD.

present similar morphology phenotypes. Nurse cells display morphological hallmarks of apoptosis, such as an irregular nuclear shape, and become positive for TUNEL staining. In contrast, follicle cells remain intact and eventually engulf the cellular remnants of the degenerated germ line before they themselves degenerate. In *Drosophila*, apoptosis is positively regulated by a set of three genes, *reaper* (*rpr*), *head involution defective* (*hid*), and *grim*, which are deleted in a single chromosome deficiency on the third chromosome, *Df(3R)H99* (Cashio et al., 2005). These genes act as upstream activators of apoptosis in various developmental contexts, such as embryo development, PCD of larval tissues during metamorphosis, or patterning of adult tissues. REAPER, HID, and GRIM proteins act by binding to and inactivating the caspase inhibitor DIAP 1 (Cashio et al., 2005). Surprisingly, however, mid-oogenesis PCD is not affected by the *H99* deficiency, indicating that these pro-apoptotic genes are not involved in this checkpoint (Peterson et al., 2007). Thus alternative, ovarian specific pro-apoptotic signals must be active in mid-stage egg chambers for the activation of caspases, the terminal effectors of PCD.

Ecdysone signaling is an important element of this checkpoint. Indeed, *Drosophila* germ line mutant clones of the ecdysone receptor degenerate in mid-oogenesis (Buszczak et al., 1999; Carney and Bender, 2000), indicating that ecdysteroid hormone is required for the

progression past this checkpoint. In addition, nutrient deprivation, which activates this checkpoint, is known to reduce the level of ecdysone in different insect groups such as Diptera and Hymenoptera.

Late-oogenesis PCD

At the end of oogenesis, nurse cells enter apoptosis after the rapid transfer of nurse cell cytoplasmic contents to the oocyte. This final transfer of material, called “dumping,” lasts about 30 minutes in *Drosophila* and follows the slow traffic of molecules that takes place during vitellogenesis (Mahajanmiklos and Cooley, 1994). Dumping itself is not responsible for the entry into apoptosis. First signs of apoptosis in nurse cells are detected at stage 10B with important actin rearrangement, change in nuclear morphology, and permeabilization of the nuclear envelope (Foley and Cooley, 1998). At the molecular level, a dramatic increase of the DREDD caspase mRNA is also observed in both nurse cells and oocyte. After dumping, the depleted nurse cells are cleared from the egg chamber during stage 12 and 13 and enter apoptosis, showing DNA condensation and fragmentation, and TUNEL labeling (Foley and Cooley, 1998).

In contrast to mid-stage PCD, late-stage PCD is only partially caspase-dependent (Baum et al., 2007). Caspase’s involvement in the nuclear destruction of nurse cells only occurs after the transfer of cytoplasm to the oocyte, probably as a way to avoid inducing oocyte death (Peterson et al., 2007). Follicle cells also enter apoptosis at the end of oogenesis after the secretion of vitelline membrane and chorion onto the mature oocyte.

In addition to apoptosis, autophagy has been recently reported to cooperate with apoptosis during mid- and late oogenesis PCD in *Drosophila virilis*, *Ceratitis capitata*, and *Bombyx mori* (Mpakou et al., 2006; Velentzas et al., 2007a; 2007b). However, the function and molecular aspects of autophagy during oogenesis remain poorly understood.

What happens in A. tabida?

Wolbachia removal in *A. tabida* does not induce a general apoptosis but a cell death restricted to mid-oogenesis egg chambers, and more precisely in the nurse cells of previtellogenic egg chambers (Figure 3.2; Dedeine et al., 2001; Pannebakker et al., 2007). Based on these cytological observations, it is probably the mid-oogenesis PCD that is massively induced in *A. tabida* when endosymbiotic *Wolbachia* are removed. This specificity of the effect suggests that *Wolbachia* could interact with the noncanonical PCD pathway that characterizes this checkpoint.

Using suppression subtractive hybridization (SSH), no over-expression of caspases could be detected in uninfected individuals (NK, unpublished results). Because autophagy is often defined as a caspase-independent form of PCD, it is possible that the observed phenotype relies on such noncanonical PCD pathways. Because the different points where PCD might be triggered are under the control of different pathways in *Drosophila* oogenesis, this is an important point and elucidating this issue should be the focus of future research.

Because the mid-oogenesis checkpoint is also dependent on external signals (like nutrient deprivation) it is still not clear whether PCD is directly under the control of *Wolbachia*, or whether induction of apoptosis is only a by-product of some other manipulation of the host. The fact that the *Wolbachia* outer surface protein (Wsp) from the nematode *Diofilaria immitis* is able to inhibit apoptosis of human granulocytes (Bazzocchi et al., 2007) and that close relatives of *Wolbachia*, such as *Rickettsia*, can manipulate the PCD of their vertebrate hosts through the NF- κ B pathway (Clifton et al., 1998) suggest that direct manipulation of

PCD is possible. Interestingly, a recent microarray study on the *Drosophila* S2 cell line has shown that the expression of immune genes, among which are NF- κ B genes, is modified by the presence of *Wolbachia* (Xi et al., 2008).

In addition, rescue of host oogenesis by *Wolbachia* has also been shown in *Sxl^{l^f}* mutants in *Drosophila melanogaster* (Starr and Cline, 2002). Although uninfected mutants are unable to mature eggs, infected females produce some, showing that becoming dependent on *Wolbachia* for oogenesis can result from mutation in a single gene. Interestingly, this situation is rather different from that in *A. tabida*, because absence of oogenesis is due to an over-proliferation of follicular cells. Further study is required to determine whether PCD is involved in the phenotype and/or in its rescue by *Wolbachia*.

In addition to the cases of *A. tabida* and *D. melanogaster*, different *Wolbachia* strains have been shown to interfere with their host oogenesis. In *Drosophila simulans*, over the course of twenty years of infection, the *Wolbachia* strain *w*Ri has evolved from reducing its host's fecundity by 15%–20% to increasing it by 10% (Weeks et al., 2007). Increase in host fecundity by infection has also been demonstrated in the wasp *Trichogramma bourarachae* (Girin and Boulétreau, 1995 ; Vavre et al., 1999), the mosquito *Aedes albopictus* (Dobson et al., 2002), and the uzyfly *Exorista sorbillans* (Puttaraju and Prakash, 2005). In addition, symbiont infection in the date stone beetle *Coccotrypes dactyliperda* (Zchori-Fein et al., 2006) seems obligate for oogenesis, but double-infection with *Rickettsia* and *Wolbachia* has not yet allowed the determination of which of these symbionts is responsible for this phenomenon. All these examples show that reproductive manipulators might frequently interfere with their host oogenesis. Because of their vertical mode of transmission, reproductive manipulators have a strong tropism for reproductive organs, which could allow for easier interactions with this developmental program.

From immunity to developmental programs: a role for pleiotropy?

As shown above, PCD has a crucial role in both immunity and development and can be used as an illustrative example of the evolutionary scenarios that may arise from pleiotropy. Induction of the immune system can have direct or indirect effects on both partners, and most notably on the host developmental program. The evolutionary trajectory of the association will depend on the outcome of the modification of the developmental program of the host, which can be: (1) negative consequences for the host; (2) benefit both for host and symbiont; (3) benefit for neither partner (Figure 3.4).

Although immune reactions are evoked to protect the host, they can have detrimental effects on various host traits. An interesting example is found in *Anopheles gambiae* where infection by its *Plasmodium* parasites induces a reproductive cost on host fecundity (Ahmed and Hurd, 2006). In this system, induction of immunity creates an oxidative burst that triggers the apoptotic pathway within the follicular cells, inducing resorption of ovarian follicles. However, when the immune system is challenged by sephadex beads, oogenesis is affected as well, suggesting that the modification of the host developmental program is a side effect of infection. The *Anopheles-Plasmodium* case is very similar to the numerous parasites that castrate their hosts, where inhibition of gametogenesis allows parasites to redirect resources to their own development. The origin and the mechanisms responsible for castration remain largely unknown, but involvement of apoptosis has been demonstrated in the castration of *Acyrtosiphon pisum* by the wasp *Aphidius ervi* (Falabella et al., 2007), and down-regulation of phenoloxydase (which is also involved in immune response) by *Schistosoma mansoni* has been suggested in the snail *Biomphalaria glabrata* (Bai et al., 1997). It is not clear yet whether these manipulations are actually linked to the

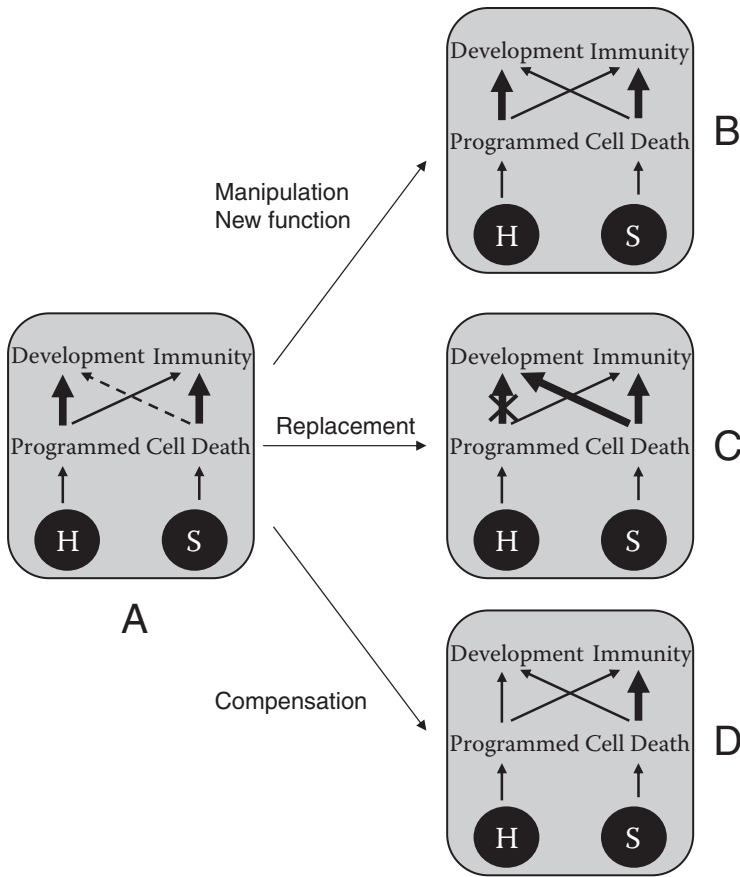


Figure 3.4 Pleiotropy of PCD in immunity and development and the evolution of symbiosis. (A) The induction of the immune system by the host (H) or its manipulation by the symbiont (S) can induce side effects on the host development (dashed arrow). This modification to the host development can become an important part of the interaction and be the target of selection with different possible outcomes. (B) The new extended phenotype can be beneficial for the symbiont (that starts to actively manipulate its host development) or both partners, in which case new functions can arise. (C) The function is no longer accomplished by the host but has been transferred to the symbiont (represented by the bigger size of the arrow for development). (D) Both the host and the symbiont are involved in the accomplishment of the function, but the expression of genes in the host has been modified to compensate for the presence of the symbiont. In (C) and (D), dependence of the host upon its symbiont can evolve despite the fact that no new function has emerged.

evasion of the host immune system. The situation in mosquitoes, however, does suggest such a link exists and the side effect shown in mosquitoes could instead be a direct manipulation by the parasite in other systems.

A different outcome is when both partners benefit from the modification. In this case, selection will favor a further integration of the physiology and genetic pathways of both partners. The best-characterized example of the molecular interplay occurring in invertebrate mutualisms is the formation of a light-emitting organ in the mutualism between the squid *Euprymna scolopes* and its environmentally acquired luminescence-inducing bacterial

symbiont, *Vibrio fischeri* (reviewed in Nyholm and McFall-Ngai, 2004). Strikingly, the pathways used for the initiation of the symbiosis and the development of the light organ are very similar to what occurs during infections with pathogens. When symbionts are taken up from the environment, they release tracheal cytotoxins (a fragment of the PGN), which act synergistically with LPS to trigger apoptosis that leads both to epithelial regression and to the morphogenesis of the light organ (Koropatnick et al., 2004). Further characterization of this system is under way and may involve the NF- κ B pathway (Goodson et al., 2005), a classical target of pathogens. A similar apoptotic regression of the organ involved in symbiont acquisition has been found in hydrothermal vent tubeworms (Nussbaumer et al., 2006). In both these cases, the molecular pathways triggered in the host are directly related to immune pathways, and it is tempting to propose that these mechanisms have evolved from the ability of these bacteria to evade the host immune system.

Finally, it is possible that neither partner benefits from the modification of the host developmental program. Hosts can react to the infection either directly by limiting symbiont progression or by limiting the negative impacts of the symbiont when resistance is too costly (tolerance or compensatory evolution). Evolution of tolerance has been the subject of numerous studies in plants where it has been shown, for example, that plants may adapt to the presence of grazers by a process called over-compensation (Agrawal, 2000). Similar compensatory evolution has probably occurred between amoeba and *Candidatus Legionella jeonii*, formerly X-bacteria, which have switched in the lab from parasitic to mutualistic symbionts in a few generations (Jeon, 2004). The same can happen in animal systems, and especially in reaction to reproductive manipulators that often reach very high prevalence. Hosts could be selected to adapt their own gene expression and physiology to the presence of their parasites. However, tolerance can rapidly lead to dependence as soon as these adaptations become fixed in the population. This could have occurred in *A. tabida*. *Wolbachia* might manipulate host PCD to evade the immune system, as could be suggested by recent data on the *Drosophila* S2 cell line (Xi et al., 2008). In turn, *A. tabida* might have adapted its expression of PCD to the presence of *Wolbachia*. This would result in a deregulation of PCD in females where *Wolbachia* are removed. Compensatory evolution does not always have to result in the shared control of a function, but could rather result in the loss of the function in one of the partners. In *A. tabida*, it seems that the host has lost the ability to control PCD during oogenesis.

Conclusion

Genes involved in host immunity are often also involved in host development (pleiotropy). Hence, any symbiont that is manipulating its host's immune system could potentially also influence its host's developmental program. The intense interactions of the symbiont with the host's immune system, combined with pleiotropy of genes involved in immunity and development, could make host development especially prone to symbiont manipulation. This effect is not limited to PCD, but because of its importance for both host immunity and development, PCD might play a crucial role in the evolution of host-symbiont interactions. More generally, pleiotropy between functions could be a major determinant of symbiosis evolution, which can result in several possible evolutionary outcomes.

However, direct and clear-cut evidence that the mechanisms allowing symbionts to evade the immune system are being used for the modification of the host developmental program is still lacking. The main reason for this is that although numerous data have been collected on the role of PCD and its manipulation by intracellular pathogens in vertebrates, virtually nothing is known on this topic in invertebrates, with the exception of

viruses. Insect models could provide unique systems to address questions that remain understudied, especially considering the diversity of interactions that insects have established with intracellular symbionts and the availability of insect model systems for which genetic tools are available. This research avenue is of a wider importance as well, given the numerous cases where intracellular human and veterinary pathogens are vectored by invertebrate hosts. *A. tabida* provides a system to study the role of PCD in the interplay between host and endosymbionts, but elucidating the exact role of PCD would benefit from the development of more model systems for which genetic and developmental tools are readily available. More generally, establishing bridges between host–pathogen and host–symbiont interactions and between immunity and development may help to cross-fertilize these fields, to orientate investigations and clarify the evolutionary origin of the molecular dialogue between a host and its associated microorganisms.

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chapter four

Pleiotropy of adaptative genes: how insecticide resistance genes mediate outcomes of symbiosis

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Introduction

As pointed out first by Fisher (1958), mutations favoring adaptation to a new environment are expected to be at a selective disadvantage in the previous environment. This is because they generally cause resource reallocation and affect metabolic or developmental processes, leading to reduced performance in some traits and cost to overall fitness (Bergelson and Purrington, 1996; Bergelson et al., 1996; Coustau et al., 2000; Davies et al., 1996; Levin et al., 2000; Roush and McKenzie, 1987; Uyenoyama, 1986). Indeed, it is widely expected that mutations conferring resistance to xenobiotics (e.g., antibiotics, insecticides, herbicides) will drive some cost to a resistant individual's fitness in a xenobiotic-free environment. This expectation is well supported by data from field and laboratory studies, involving a broad range of target organisms and a diversity of toxics deployed against them (Andersson and Levin, 1999). Fisher's prediction has been verified for herbicide, pathogen, and herbivore resistance in plants (reviews in Bergelson and Purrington, 1996; Simms and Rausher, 1987; Simms and Triplett, 1994), for antibiotic resistance in bacteria (review in Levin et al., 2000), and for pesticide resistance in arthropods (reviews in Coustau et al., 2000; Roush and McKenzie, 1987). Monitoring of natural populations indicates that resistance genes spread and increase in frequency in treated areas and are strongly selected against in adjacent nontreated areas, as they do not displace susceptible genes (Lenormand et al., 1999). This indicates a substantial fitness cost, which later may be compensated for

by secondary mutations (Fisher, 1958; Levin et al., 2000). Laboratory experiments generally demonstrate that numerous life-history traits are modified in resistant insects, including increased development time, reduced predation avoidance, reduced reproductive success, and reduced survival (Agnew et al., 2004; Bergelson et al., 1996; Berticat et al., 2002a, 2004; Duron et al., 2006c; Foster et al., 2005), consistent with the high fitness cost measured in natural populations.

Interactions with other organisms impose additional constraints on the rates of population growth. Symbiotic organisms are of particular interest because they represent a large proportion of known biological diversity and a major source of selection acting in natural populations (Price, 1980). Symbiotic associations are very common and the various degrees of relationship between different organisms encompass a continuum from mutualism to parasitism. Because the evolution of symbionts and their hosts are linked, each partner of the association potentially exerts a selective pressure on the other. The factors that control replication rate of symbionts are generally unknown and difficult to explore, given the complex interaction of symbiont and host genotypes. In a more general way, coevolution is a logical consequence of symbiotic associations and even a small change in the host's metabolism could interfere with host-symbionts relationships and thus modulates the outcome of the symbiosis.

Because insecticide resistance genes generate extensive variations in insect physiology, they provide an interesting opportunity to study how insect symbiosis evolves, specifically the evolution of host resistance, parasite virulence, and transmission dynamics. Insecticide resistance is one the most fascinating cases of microevolution that have been thoroughly investigated as an opportunity to assess precisely both the new adapted phenotypes and the associated genetic changes. A noticeable feature is that resistance is achieved with few genes, and these genes generally have a large physiological effect. We have recently established that insecticide resistance genes finely modulate relationships between the common house mosquito *Culex pipiens* and its intracellular symbiont, the alpha-proteobacterium *Wolbachia*, a process that mediates the outcome of this symbiosis (Berticat et al., 2002b; Duron et al., 2006c). A likely interpretation of this result is that susceptible mosquitoes normally invest in *Wolbachia* density control, but when bearing the physiological cost of resistance, are less able to do so. As a result, polymorphism in traits affecting outcomes may be maintained through conflicting selection pressures. We present in this chapter how variations in outcome of symbiosis can be maintained within populations through the pleiotropic action of insecticide resistance genes. Pleiotropy means that an isolated gene has more than one distinguishable phenotypic effect, e.g., insecticide resistance and control of symbiont density in mosquitoes. Our aim is to provide both mechanistic and evolutionary backgrounds that could explain how adaptations to abiotic environment lead symbiosis toward a new evolutionary step. First, we introduce the genetic and metabolic basis of insecticide resistance and the fitness cost of insecticide adaptation. Second, we consider the role of insecticide resistance in the evolution of symbiosis. We stress throughout the relevance of our findings with the help of different symbiotic associations in mosquitoes.

Insecticide resistance

Basis of insecticide resistance

The wide use of pesticides to control pests of agricultural and public-health importance has been a powerful and recent agent of selection in natural populations. The *C. pipiens* mosquito, common in temperate and tropical countries, is subjected to insecticide control in

many places, particularly with organophosphate insecticides (OP), because it is a nuisance and a vector of human diseases (West Nile encephalitis, filariasis, etc). The OP insecticides inhibit the acetylcholinesterase (or AChE) in the central nervous system, inducing lethal conditions. This mosquito has rapidly developed various adaptations to these new and toxic compounds of its environment. The genetic basis of OP resistance involves independently the super-locus *Ester* (detoxification processes) and the locus *ace-1* (target protein), both displaying major resistance alleles (Raymond et al., 2001).

The super-locus *Ester* includes two loci (*Est-2* and *Est-3*) separated by an intergenic DNA fragment varying between 2 and 6 kb, and codes for detoxifying carboxylester hydrolases (or esterases). Several resistance alleles (each corresponding to a distinct over-produced allozyme) have been described at each *Ester* loci (Raymond et al., 2001). They correspond to an esterase over-production (which binds and/or metabolizes the insecticide) relative to basal esterase production of susceptibility alleles. Overproduced esterases may account for up to 12% of the soluble proteins of resistant individuals, and are less than 1% in susceptible mosquitoes (Fournier et al., 1987). This overproduction is the result of two non-exclusive processes. The first one is a gene amplification (i.e., several copies of the same gene are found in the same genome) and concerns either one *Est* locus (for instance, the *Ester^{B1}* allele) or both loci (*Ester²* or *Ester⁴* allele). Furthermore, the level of gene amplification varies between the different amplified alleles from a few copies to 100 copies and also among populations for a given amplified allele (Weill et al., 2000). The second process of overproduction is a gene regulation (*Ester¹* allele). The overproduction occurs in tissues lining the cuticula, the digestive tract, and Malpighian tubules, but it varies in other tissues depending on alleles involved, in particular in the brain and ganglia where the insecticide target is located. This may change the efficiency of each amplified allele to reduce the insecticide sensitivity at the target location (Pasteur et al., 2001).

The third locus, *ace-1*, codes the insecticide target AChE1 (Weill et al., 2002, 2003, 2004). The susceptible form of this enzyme is inhibited by OP insecticides. The resistance allele *ace-1^R* codes for a mutated AChE1 that is less inhibited by OP (Weill et al., 2003), but is associated with a 60% reduction in activity compared to the susceptible enzyme (Bourguet et al., 1997). Thus, modification of AChE1 results in an excess of acetylcholine (ACh) in synapses impairing the optimal functioning of the signal transmission. Several particular resistance mutants have been recently described (*ace-1^D*), corresponding to a duplication of the gene *ace.1* harboring one susceptible and one resistant copy on the same chromosome (Lenormand et al., 1998; Labbé et al., 2007a, 2007b).

Additional loci are probably contributing to OP resistance, such as genes involved in mono-oxygenase detoxification or reduced penetration (Raymond et al., 1987, 1989). However, their contribution for resistance to OP is relatively minor in *C. pipiens* compared to overproduced esterases or target insensitivity (cf. Raymond et al., 2001).

Fitness cost of resistance

Insecticide resistance genes are widely distributed in *C. pipiens* over the planet, as it has been reported in most places investigated. Due to the advantage they provide in OP treated areas, these resistance genes have subsequently spread within populations, and then among populations, a process considerably facilitated by passive migration via human activities (Raymond et al., 2001). Some resistance alleles such as *Ester²* are now found on several continents, after their first occurrence in one geographic location probably in the 1960s (Labbé et al., 2005). Their actual geographic distribution undoubtedly corresponds to areas where OP insecticides are regularly applied. However, Fisher's prediction (Fisher, 1958) assumes

that insecticide resistance genes responsible for adaptation to the toxic environment have a fitness cost, i.e., are at a disadvantage in the insecticide-free environment. This prediction is well supported by the decrease of OP resistance genes whenever OP insecticides are no longer used, even within a year. Indeed, insecticide selection varies through the year in temperate areas: first and last breeding generations (as well as the over wintering generation) generally escape OP treatments. A high survival cost associated to OP resistance occurs during these periods without OP selection, especially during the over wintering period (Chevillon et al., 1997; Gazave et al., 2001). The distribution of the resistance allele *Ester*², which has a broad geographic distribution compared to the other resistance alleles, provides more evidence of fitness cost, as pointed out by Labbé et al. (2005). In some areas, OP insecticides were replaced by *Bacillus sphaericus* toxin, and *Ester*² (which does not confer resistance to the bacterial toxin) disappeared a few years after the first report of occurrence (Eritja and Chevillon, 1999). *Ester*² was reported from places across Italy where OPs were continuously used, but decreased rapidly in frequency and was not detected a few years after OP treatments had been stopped (Silvestrini et al., 1998). All suggest that a fitness cost is associated with *Ester*², thus precluding the presence of *Ester*² at high frequencies in nontreated areas.

Resistance to pesticides, and in particular resistance to OP in *C. pipiens*, has been extensively studied in the Montpellier (France) area for more than 30 years and provides the most detailed survey to date. The Montpellier area is the subject of a long-term longitudinal study for the evolution of the resistance genes since 1972 (Labbé et al., 2005). OP treatments started in 1968, and were restricted to a 20–25 km wide belt along the coast. Resistance first appeared in 1972 with the occurrence of *Ester*¹, followed by *ace-1*^R in 1978, *Ester*⁴ in 1984, *Ester*² in 1990, and *ace-1*^D in 1993 (Guillemaud et al., 1998; Raymond et al., 2001). Resistance genes spread and increased in frequency in the treated area, and also migrated into the nontreated area, where they were selected against due to their fitness cost. A decline of frequency observed across the treated and nontreated areas is the result of a balance between selection (i.e., fitness advantages and costs) and migration (Labbé et al., 2005; Lenormand et al., 1999; Lenormand and Raymond, 2000). Resistance genes decline in frequency along transects from treated to nontreated areas, arguing for a strong fitness cost associated to OP resistance in natural populations. Laboratory experiments, using *C. pipiens* strains sharing the same genetic background, showed that resistance genes alter fitness-related life history traits, including increased larval development time and preimaginal mortality, and reduced male reproductive success, female size, and fecundity, relative to susceptible genes (Agnew et al., 2004; Berticat et al., 2002a; Duron et al., 2006c). The outcomes of symbiosis also change according to mosquito genotype at the insecticide resistance loci and is further explained in the section “Mediation of Symbiosis.”

How insecticide resistance genes generate fitness cost could be explained by the alteration of the mosquito physiology. The overproduction of esterase by the *Ester* locus should be at the expense of producing something else. Thus an overproduction is likely to be costly *de facto* and could also drive severe physiological disorders. Indeed, the esterase genes involved in resistance in other pest species, such as the sheep blowfly *Lucilia cuprina*, have apparently several functions, as they also operate during embryogenesis and metamorphosis (Clarke et al., 2000; Davies et al., 1996). The modified AChE1 alters the optimal functioning of cholinergic synapses of the nervous system, because of reduced catalytic properties for the natural neurotransmitter, with deep changes in some physiological, but also behavioral, traits. Furthermore, this enzyme is also involved in the development of the nervous system in vertebrates and invertebrates (Grisaru et al., 1999; Cousin et al., 2005).

Insecticide resistance genes affect mosquito behavior and greatly reduce predation avoidance, increasing the probability of predation, at both the larval and the adult stages (Berticat et al., 2004). *C. pipiens* larvae are able to detect chemicals released by conspecifics that have been preyed upon by backswimmers, and adjust their behavior to reduce the predation risk by choosing a less risky microhabitat (a vegetation refuge, the edge of the breeding site, etc.) and moving less (Sih, 1986). Larvae bearing the *ace-1^R* resistance gene display a distinct feeding behavior, as they replace their gut contents at a faster rate than the other strains (Agnew et al., 2004). This observation is consistent with the hypothesis that resistant larvae are more active, and then more easily detected by predators and killed. Adult mosquitoes with *Ester* resistance gene also have a higher predation probability by pholcid spiders, which suggests that they either are more active (thus with a higher probability of flying near the web or the spider) or have fewer chances to escape an attack. These results indicate that insecticide resistant genes deeply alter the interactions with other organisms and are subject to counterbalanced selection via biotic interactions.

Cost is really important in the evolution of adaptation because it can lead to allelic replacement (an allele is replaced by a less costly one) or to selection of modifier genes. Estimations of overall fitness costs from population surveys have shown that *ace-1* is associated with higher deleterious effects than *Ester* (Lenormand et al., 1999; Lenormand and Raymond, 2000). At the *Ester* super-locus, the metabolic cost of this overproduction varies depending on the alleles involved (Agnew et al., 2004; Berticat et al., 2002a, 2004; Duron et al., 2006c). For example, preimaginal mortality was lower for *Ester²* than *Ester⁴* or *Ester¹* individuals. Similarly, preimaginal mortality was lower for *Ester⁴* than *Ester¹*. These results are in agreement with field surveys in the Montpellier area, which show that *Ester⁴* replaced *Ester¹* during the 1980s, and that *Ester²* is now increasing in frequency (Guillemaud et al., 1998; Labbé et al., 2005; Lenormand et al., 1999). This pattern of replacement clearly indicates that *Ester²* enjoys a competitive advantage, at least locally, over previously prevalent resistance alleles. A similar phenomenon was observed at the *ace-1* locus: the *ace.1^D* allele quickly increased in frequency after 1993 (Lenormand et al., 1998) but it did not replace *ace.1^R* in the Montpellier area because of a deleterious effect in the homozygous state (Labbé et al., 2007b). For the *ace.1^D* allele, the additional S copy does not modify the resistance provided by the R copy, thus its advantage is probably a lower cost, as the additional AChE1 activity provided by the S copy probably compensates for deficiency of AChE1 activity of the R copy. Therefore, the S copy can be considered as a modifier for the cost generated by the R copy.

Mediation of symbiosis

Insecticide resistance obviously modifies the mosquito physiology and has been reported to influence the interactions with at least three parasites inhabiting *C. pipiens*, the alpha-proteobacterium *Wolbachia* (Berticat et al., 2002b; Duron et al., 2006c), the microsporidia *Vavraia culicis* (Agnew et al., 2004), and the filarial worm *Wuchereria bancrofti* (McCarroll et al., 2000). Because insecticide resistance appeared recently, resistant mosquitoes offer a new physiological environment to parasites, in which they could be less adapted relative to an ancestral host (i.e., insecticide susceptible mosquitoes).

Wolbachia symbiosis

Wolbachia is a maternally inherited bacterium commonly found in arthropods (over 16% of arthropod species are infected; Werren and Windsor, 2000) that invades host populations

through strategies referred to as reproductive parasitism (Bandi et al., 2001; Stevens et al., 2001; Stouthamer et al., 1999; Werren, 1997). *Wolbachia* promotes the production and fitness of infected daughters (i.e., the transmitting sex). Because males represent dead-end, manipulations frequently involve biasing sex ratio (SR) in the offspring of infected females toward the production of daughters. *Wolbachia* induces either thelytokous parthenogenesis (production of female progeny from unfertilized eggs), feminization of genetic males, or male-killing. In a large number of hosts, such as *C. pipiens*, *Wolbachia* rather induce cytoplasmic incompatibility (CI), which is sterility between infected males and uninfected females or between individuals carrying different types of cytoplasmic infection. The death of progeny from uninfected females confers a reproductive advantage to infected females, enhancing the spread of infection (Rousset and Raymond, 1991). The spread of a CI-*Wolbachia* within an arthropod population corresponds to a balance between the bacterial transmission efficiency, the intensity of incompatibilities (i.e., the proportion of abortive embryos), and the cost suffered by infected individuals (Hoffman and Turelli, 1997). There is generally considerable variability (parasitism to mutualism) among *Wolbachia*/host interactions (e.g., Dedeine et al., 2001; Dobson et al., 2002; Girin and Boulétreau, 1995; Min and Benzer, 1997; Poinot and Mercot, 1997; Turelli and Hoffmann, 1995; Vavre et al., 1999), thus it is not surprising to find that *Wolbachia* reduces fitness in some hosts (Girin and Boulétreau, 1995; Min and Benzer, 1997). *Wolbachia* have substantial impacts on host phenotypes, altering insect sex ratios, favoring loss of sexual reproduction, driving genome evolution, and possibly promoting speciation (Bandi et al., 2001; Hurst and Jiggins, 2005; Hurst and Werren, 2001; Stevens et al., 2001; Stouthamer et al., 1999; Werren, 1997, 1998). Overall, reproductive parasites, such as *Wolbachia*, are now regarded as very important cryptic drivers of arthropod ecology and evolution.

C. pipiens is naturally infected by *Wolbachia* and worldwide prevalence surveys have shown that *Wolbachia* infection is fixed in natural populations (Duron et al., 2005; Rasgon and Scott, 2003), although a few uninfected populations were identified on the border of the host distribution area (Cornel et al., 2003; Rasgon et al., 2006). Berticat et al. (2002b) have measured the *Wolbachia* density in susceptible and resistant mosquitoes using real-time quantitative PCR assay. They used a large set of strains, all homozygous for one resistance allele at one of the two insecticide resistance loci, introgressed with the same cytoplasmic (including *Wolbachia*) and nuclear genomes, through repetitive (more than 14) backcrosses. Because these strains share the same genetic background and the same *Wolbachia* strain, all the differences observed between strains involved the genotype at the insecticide resistant loci. *Wolbachia* density was strongly increased by the presence of insecticide resistance genes in laboratory strains and in field samples of *C. pipiens* pupae (Berticat et al., 2002a) (Figure 4.1). Increased bacterial density was observed for the two distinct resistance mechanisms to OP insecticides (i.e., increased detoxification via *Ester* superlocus and target insensitivity via *ace-1* locus), suggesting that perturbations of host physiology at a global level induce a higher susceptibility to *Wolbachia*. Of particular interest is that insecticide resistance genes, *Ester* and *ace-1*, are then *Wolbachia* susceptible genes in *C. pipiens*. Insecticide resistance genes are new for the mosquito *C. pipiens*, as they appeared during the 1970s (Raymond et al., 2001), and susceptible insecticide genes represent ancestral status that have coevolved with the whole organism and its symbionts. In *C. pipiens*, *Wolbachia* may be found in most host tissues (Dobson et al., 1999), unlike most insects where they are concentrated in gonads. However, presently *Wolbachia* density has been studied only on whole insects. Knowing that the tissue location of overproduced esterases varies according to the *Ester* allele (Pasteur et al., 2001), it will be worthwhile to investigate how this factor affects *Wolbachia* associated cost.

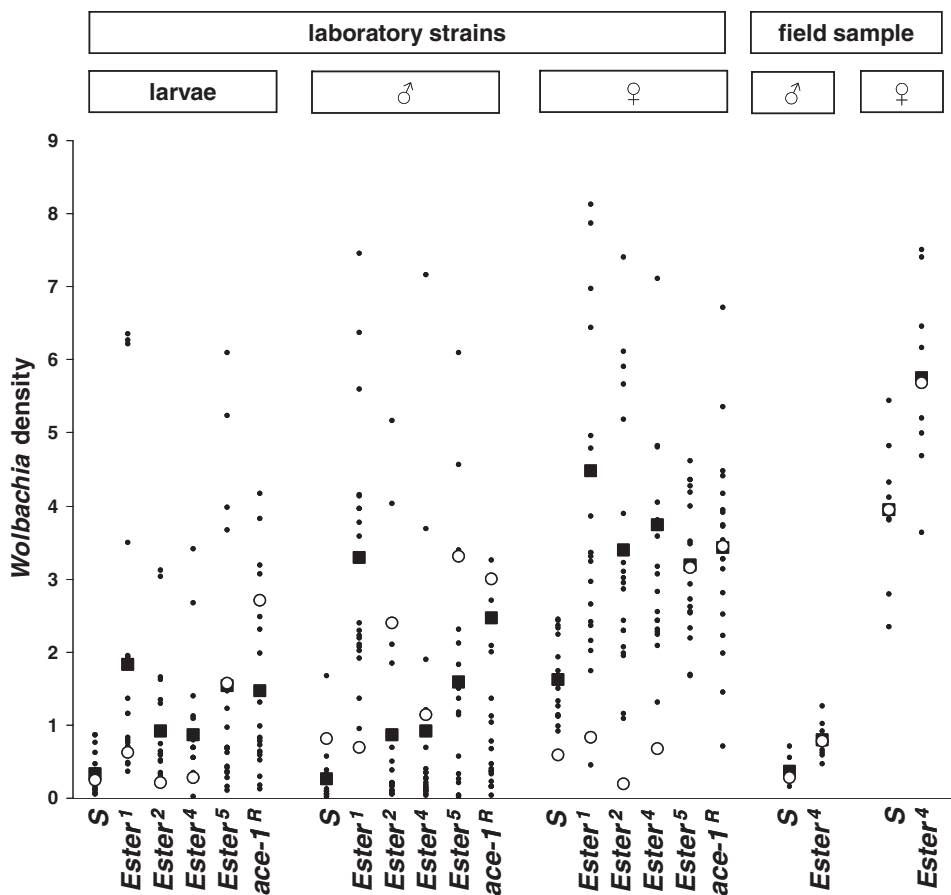


Figure 4.1 Variations in *Wolbachia* density among insecticide susceptible and resistant *Culex pipiens* mosquitoes from laboratory strains and field sample. The comparison involves mosquitoes without insecticide resistance genes (S) and mosquitoes bearing only one of the resistant genes (*Ester*¹, *Ester*², *Ester*⁴, *Ester*⁵, or *ace-1*^R). Laboratory strains are homozygous for resistance genes and share the same genetic background, including *Wolbachia*. All measures are performed on fourth-instar larvae or 5-day-old males and females. *Wolbachia* density was estimated by real-time quantitative PCR assay and corresponds to the number of *Wolbachia* genomes relative to *Culex* genomes. Each point refers to the mean of a triplicate measure of one individual. The squares and circles refer to the means and medians, respectively, of the distribution of individual measures. In all cases, insecticide resistant mosquitoes are significantly more infected than susceptible mosquitoes. *Wolbachia* density also varied according to sex (higher in females than in males) as observed in other insects and may therefore be explained by the much larger size of the ovaries relative to the testes. (Adapted from Berticat et al. 2002b.)

Cost associated to *Wolbachia* infection appears to be generally related to their density, suggesting that virulence is linked to bacterial population size (Duron et al., 2006c). A relationship between density and virulence has been documented by McGraw et al. (2002) in *Drosophila simulans* and by Mouton et al. (2004) in the wasp *Asobara tabida*, both studies demonstrating that the cost associated with *Wolbachia* infection was reduced when density decreased. The increase of *Wolbachia* density reported in resistant mosquitoes suggests

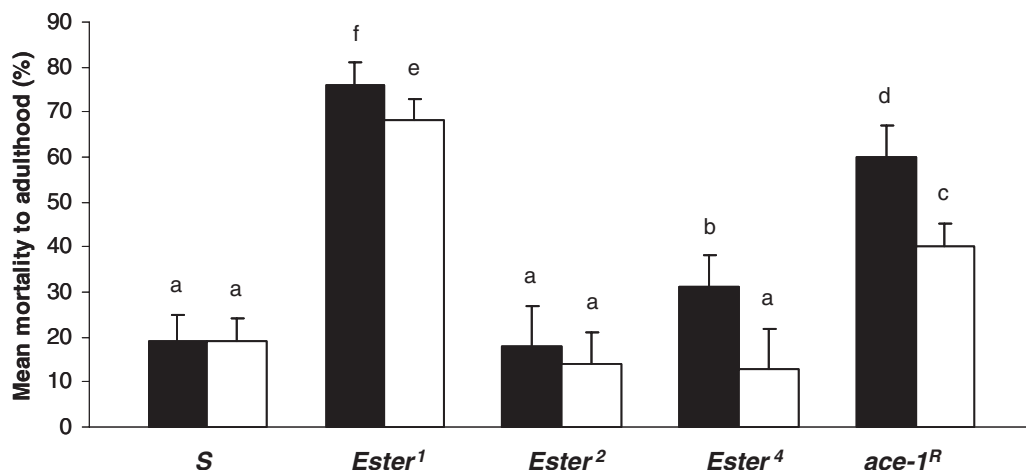


Figure 4.2 Effects of *Wolbachia* infection on preimaginal mortality in laboratory strains of *Culex pipiens*. The comparison involves homozygous strains without insecticide resistance genes (S) or bearing only one of the resistant genes (*Ester*¹, *Ester*², *Ester*⁴, or *ace-1*^R) and reared at low larval density. Black boxes represent infected strains and empty boxes, uninfected strains; a, b, c, d, e, and f represent statistic groups. *Wolbachia* display an infection cost only in strains homozygous for *Ester*¹, *Ester*⁴, and *ace-1*^R. (Adapted from Duron et al., 2006c.)

that perturbations of host physiology in a general sense play a major role in *Wolbachia* susceptibility. Experiments comparing life history traits of infected and uninfected mosquito strains sharing a similar genetic background, except for the presence or absence of resistance alleles and/or *Wolbachia* infection, revealed no host fitness advantage due to *Wolbachia*, and outcomes ranged from commensalism to parasitism (Duron et al., 2006c). For the insecticide susceptible mosquito strain (less infected by *Wolbachia*), no difference in life-history traits was detectable when *Wolbachia* were removed (Figures 4.2 and 4.3A). However, the cost of infection (reduction in size, fecundity, and preimaginal survival) was obvious in almost all strains carrying insecticide resistance alleles at either *Ester* or *ace-1* loci (Figures 4.2 and 4.3B). In terms of infection cost, *Wolbachia* infection appeared asymptomatic in susceptible mosquitoes (i.e., in ancestral host types) because of lower density, but costly in recently appeared insecticide-resistant mosquitoes.

The increase of density and cost of infection in resistant mosquitoes could limit the invasiveness of *Wolbachia*. The classical models of CI dynamics (Hoffman and Turelli, 1997; Turelli, 1994) suggest that significant reductions in host fitness may be stable in a population if they are linked to gains in *Wolbachia* transmission. A formal prediction is that *Wolbachia* will minimize their cost by reducing their density in order to maximize maternal transmission (McGraw and O'Neill, 1999). However, a strong transmission efficiency (>99% of the progeny from infected females are infected) occurs in insecticide susceptible mosquito strains demonstrating that a low density (relatively to those in insecticide resistant mosquito strains) is enough to transmit almost perfectly *Wolbachia* to the progeny (Duron et al., 2006c). Furthermore, *Wolbachia* loads show no major effect on CI expression in *C. pipiens*: no variation in hatching rates was detected, whatever the *Wolbachia* density in males (Duron et al., 2006c). CI was complete in incompatible crosses (i.e., no hatching) between insecticide susceptible males (weakly infected) and uninfected females. All crosses performed

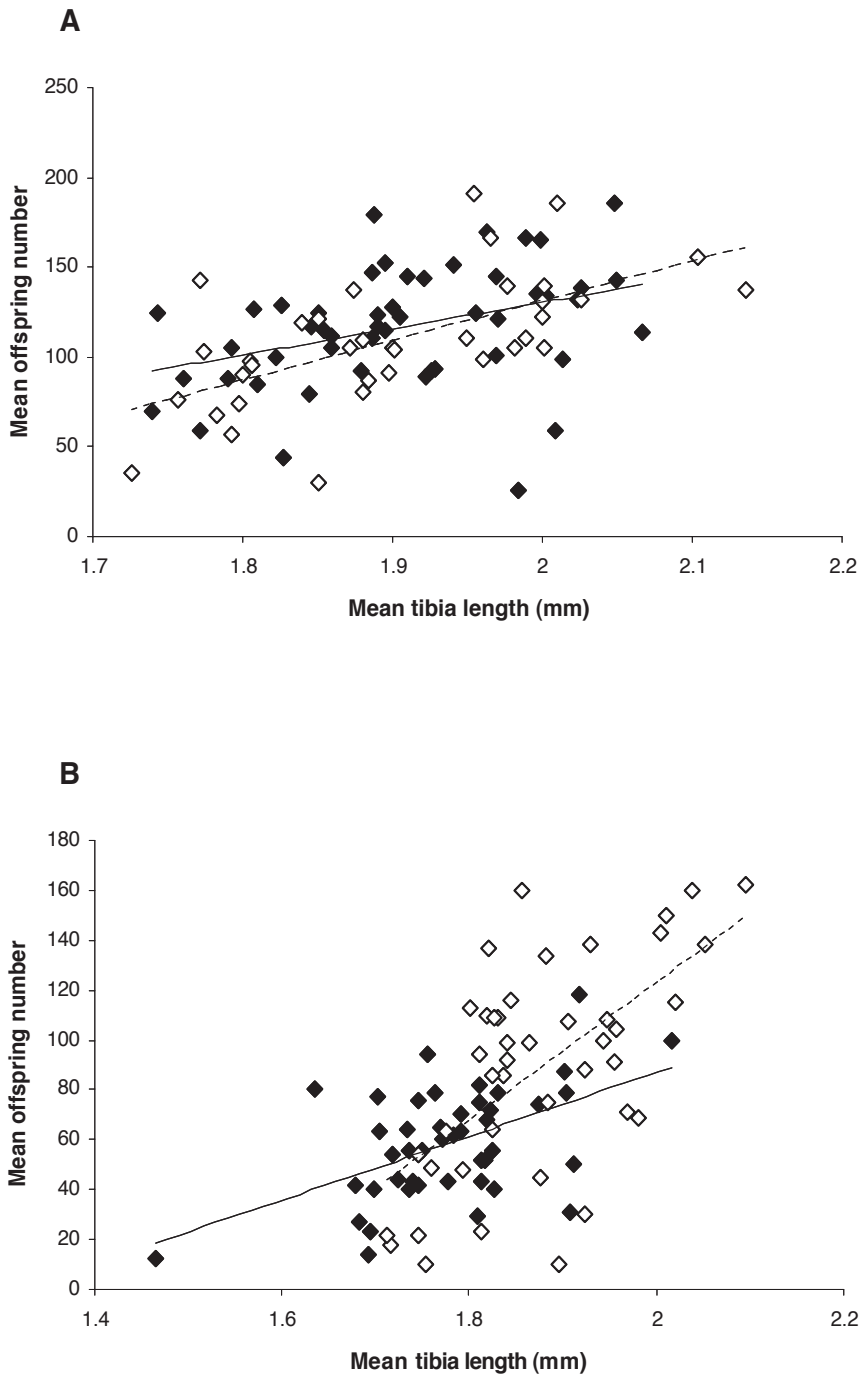


Figure 4.3 Effect of *Wolbachia* on mean tibia length and fecundity of *Culex pipiens* females. The comparison involves homozygous strains without insecticide resistance genes (A) or bearing only the *ace-1^R* resistance gene (B). Filled squares and solid lines represent infected individuals whereas empty squares and dotted lines, uninfected individuals. In all cases, female fecundity is related to the tibia length which indicates the body size. Significant effect of *Wolbachia* infection on tibia length and fecundity was associated only to the *ace-1^R* resistance gene. (Adapted from Duron et al., 2006c.)

with insecticide resistant males (highly infected) were identical to those performed with susceptible males; no hatching rate decrease was observed. This result contrasts with studies in other insects where reduced CI expression correlated with reduced bacterial densities in males (Clancy and Hoffmann, 1998; Noda et al., 2001; Sinkins et al., 1995; Bourtzis et al., 1996; Poinso et al., 1998; Veneti et al., 2003). However, even if density increases in the insecticide resistant mosquito, one cannot definitely rule out a threshold effect, above which CI rate no longer correlates with density (Duron et al., 2007), as proposed for the wasp *Leptopilina heterotoma* (Mouton et al., 2006). It is possible that *Wolbachia* density in *C. pipiens* males is always sufficient to induce complete CI. In addition, bacterial density does not appear to contribute to CI strength, compared to the major influence of the endosymbiont genotype (Duron et al., 2006a, 2007). Finally, nuclear factors strongly modulating CI expression appear to be rare: it was reported once in *C. pipiens* (Sinkins et al., 2005) but not found in many other investigations (Barr, 1966; Duron et al., 2006a; Ghelelovitch, 1952; Irving-Bell, 1983; Laven, 1967). This stresses the exclusive *Wolbachia* determinism of CI expression in most *C. pipiens* populations rather than interplay between nuclear (including insecticide resistant genes) and cytoplasmic determinants. Thus, factors implicated in CI expression correspond to qualitative variables (i.e., *Wolbachia* genotype or, more rarely, host restorer genes), and apparently not to quantitative variables (i.e., *Wolbachia* density).

The high *Wolbachia* density observed in insecticide-resistant mosquitoes is thus apparently maladapted. Further evidence suggests that this situation is still evolving. Variability in infection cost between insecticide resistance alleles reflects different outcomes of the host–*Wolbachia* association. The insecticide resistant strain carrying the *Ester*² allele represents an exception among other insecticide resistant strains because no cost of infection was detected (Duron et al., 2006c) (Figure 4.2). This absence of cost suggests a peculiar interaction between *Wolbachia* and the *Ester*² allele. Interestingly, *Ester*² has the broadest geographic distribution and has invaded regions where other *Ester* resistance alleles were present (Labbé et al., 2005). For example, the strain carrying the *Ester*¹ allele is the most *Wolbachia*-infected (Berticat et al., 2002b) (Figure 4.1) and suffers the lowest preimaginal survival (Duron et al., 2006c) (Figure 4.2). Field surveys have shown that *Ester*¹ has strongly decreased in frequency, whereas *Ester*² is now increasing (Guillemaud et al., 1998; Labbé et al., 2005; Lenormand et al., 1999). These observations suggest that absence of *Wolbachia* infection cost may explain, at least partially, the lower resistance cost of *Ester*². However, there is still a fitness cost associated with resistance genes when *Wolbachia* are removed. Eliminating *Wolbachia* did not increase the performance of insecticide resistant individuals in predation avoidance or mating competition (Duron et al., 2006c). In conclusion, *Wolbachia* infection induces an additional cost of resistance for particular traits. Due to the fact that resistant mosquitoes appear unable to control *Wolbachia* loads, the fitness cost of resistance is amplified by interactions with *Wolbachia*.

Microsporidia symbiosis

Microsporidia are obligate eukaryotic symbionts closely related to those of fungi. Their adaptation to endosymbiosis is marked by a general loss of cytological complexity and by a reduction in size of their genomes, a rarely observed phenomenon in other parasitic eukaryotes (Agnew et al., 2003). Microsporidia are among the most common parasites of arthropods but also infect a large panel of other organisms. According to the association considered, their life cycles are direct or not, and they are transmitted purely vertically or purely horizontally, with some systems having both ways of transmission and two kinds of hosts (e.g., intermediate and definitive hosts).

The microsporidium *Vavraia culicis* infects the larvae of several species of mosquitoes, including *C. pipiens*, across the world. This parasitic species has a direct life cycle in which transmission success mainly relies upon infected hosts being killed as larvae or pupae, i.e., before the imago leaves the aquatic environment (Agnew et al., 2004). *V. culicis* produces typically small spores resistant to lethal environmental conditions, such as desiccation or cold temperature. Spores are ingested by mosquito larvae during feeding, and epithelial cells of the host gut are the most exposed to microsporidia infection. When the spores detect adequate conditions, they externalize a polar tube able to pierce adjacent host cells to enter directly within the host cytoplasm (Agnew et al., 2003). The content of the spore is next injected within the cytoplasm and a virulent intracellular development occurs. Proliferation of microsporidia produces a large amount of spores until the rupture of the host cell. The new generation of spores disperses into the gut lumen and either reinfects other epithelial cells or gets passed out with feces. Parasitism by *V. culicis* is costly for mosquito fitness and induces a high mortality, especially among the individuals with the slowest preimaginal development (Agnew et al., 1999). The number of produced spores is closely correlated with the proportion of hosts killed before leaving the aquatic environment, and thus determines the transmission success of *V. culicis* (Agnew et al., 2004).

Agnew et al. (2004) have tested the effects of insecticide resistance mutations on life-history traits of *C. pipiens* when infected by *V. culicis*. They used the same *C. pipiens* strains that were used in experiments described previously (cf. section “*Wolbachia* Symbiosis”). Although the overall effect of *V. culicis* was to reduce mosquito fitness, traits linked with the parasite transmission success varied among mosquito strains. Whereas the *ace-1^R* resistance allele is associated with a significantly lower probability of reaching adulthood and lower adult longevity when uninfected, the difference with the susceptible allele *ace-1^S* disappeared when infected by *V. culicis*. In contrast, the difference between *ace-1^S* and the *Ester¹* resistance allele increased in the presence of parasitism, suggesting a greater virulence of *V. culicis* in mosquitoes carrying *Ester¹*.

There are also significant differences in the amount of *V. culicis* spores harbored by the different mosquito strains (Agnew et al., 2004). Individuals with the *ace-1^R* resistance allele produce fewer spores than susceptible mosquitoes, suggesting that insecticide resistant hosts would offer less transmission success than susceptible mosquitoes. Agnew et al. (2004) suggested that differences in the number of spores produced by the different *C. pipiens* genotypes varied not because of different growth rate within hosts, but because of variation in the size of initial infections. Indeed, mosquito strains varied in their feeding behavior, and thus in their exposure to spores of *V. culicis*: the longer they feed, the more they ingest spores, increasing larval mortality. Mosquito larvae with the *Ester¹* allele have the slowest feeding rate and display the largest cost of *V. culicis* infection, whereas those with the *ace-1^R* allele feed faster, are less killed by infection, and produce fewer spores. Such variations in the parasite fitness could directly modify the invasive capacities and influence the parasite prevalence.

Filarial symbiosis

Filarial nematodes (family Onchocercidae) are arthropod-borne parasites of vertebrates responsible for major human health problems in developing countries. The subspecies *C. p. quinquefasciatus* is the principal vector of a lymphatic filariasis caused by the parasitic worm *Wuchereria bancrofti* that is endemic in some Asian countries. *W. bancrofti* are typical mosquito-borne parasites, acquired from blood-feeding on a previously infected human, and must pass through several mosquito tissues, such as gut and salivary glands, to com-

plete their development. Almost 80% of the mosquitoes are infected with *W. bancrofti* in Sri Lanka. Although never demonstrated, it is likely that *W. bancrofti* infection severely damages the mosquito host as do other parasitic organisms and then decreases the mosquito fitness.

McCarroll et al. (2000) collected blood-fed females *C. p. quinquefasciatus* from the field and analyzed them for *W. bancrofti* load as well as for metabolic insecticide resistance estimated by the level of esterases activity. In all the sampled localities, there was a strong negative correlation between esterase activity and the parasitic load. Laboratory experiments of artificial blood-feeding infection of insecticide susceptible and resistant females confirmed this pattern. Most susceptible mosquito females produced stage-L3 *W. bancrofti*, whereas no parasite was observed in any of the insecticide resistant mosquito females. Increased esterase activity seems to alter the development of *W. bancrofti* larvae, and thus confers a major resistance against two strong and common selective forces acting on *C. pipiens*: insecticides and filarial worms. High concentration of esterases found in the gut epithelial cells of resistant mosquitoes might change the cell metabolism (Pasteur et al., 2001), and could limit the growth of filarial worm in insecticide resistant *C. pipiens*. As mosquito-borne parasites must pass through tissues over-expressing esterases to complete their development, it is possible that *W. bancrofti* survival, and hence the vectorial capacity of *C. pipiens*, may be affected by the insecticide resistance. By feedback, the spread of *Ester* resistance alleles may therefore be influenced by selection pressures for both insecticide resistance and reduction of the *W. bancrofti* burden.

Concluding remarks

Of particular interest is that parasites and insecticide resistance generate an evolutionary arms race that provides a system to study the genetics of adaptation in natural environments. At least in the mosquito *C. pipiens*, insecticide resistance genes are influencing the outcome of the host-parasite associations via their pleiotropic effects. As insecticide resistance genes increase or decrease the cost of infection according to the parasite considered, the invasion dynamics of these parasites can be strongly impaired or enhanced. Alternatively, the selective pressures exerted by parasites would influence the dynamic of insecticide resistance in the host. It is worthy to note that insecticide resistance genes could be assimilated to “parasite susceptible” genes for *Wolbachia* infection but also to “parasite resistant” genes in some cases as filarial infection. Therefore, the selection acting on resistance mutations due to parasitism varies as a consequence of how they interact with the physiological phenotype associated to esterases over-production or modification of AChE1 catalytic properties. All the works on this topic consider the parasites as genetic clones and conducted comparisons of adaptive traits at the interspecific level. Nevertheless, the reality is quite different because parasites, and their hosts, are also variable at the intraspecific level. For example, more than 60 *Wolbachia* variants have been genetically identified among *C. pipiens* populations through the world, many populations being themselves infected by two or more variants (Duron et al., 2006b) that display frequently variable CI properties (Duron et al., 2006a) and probably variable costs of infection. It is likely that the cost of infection could vary between these different variants, and *Wolbachia* symbioses may vary in their outcomes to a larger extent than expected by the present studies.

The effects of insecticide resistance genes on parasites are not limited to *C. pipiens* and should be extended to a wide range of organisms. A large number of insects, such as mosquitoes, are subjected to insecticide control in many countries, particularly with OP, carbamates, pyrethroids, or DDT. Within a few years, many pests have developed various

adaptations to these toxic environments. Resistant insect species have increased greatly in frequency and geographical extent in the last few decades. Mutations conferring resistance to insecticides have been reported to involve some costs to a resistant individual fitness in a variety of arthropod species (Davies et al., 1996; Roush and McKenzie, 1987; Uyenoyama, 1986) and are likely to interact with parasites. For example, the insecticide resistance in the *Myzus persicae* aphid induced by esterases overproduction and *kdr* mutation is associated with a greatly reduced response to the alarm pheromone, (E)- β -farnesene which is normally released by aphids when disturbed by natural enemies such as parasitoids and predators (Foster et al., 2005). Release of the pheromone causes neighboring aphids to disperse away from the pheromone source, and then from a lethal risk. Because of their action on the aphid behavior, the insecticide resistant genes mediate against interspecific interactions by increasing the risk of parasitism by parasitoids (Foster et al., 2005). Fitness studies of insecticide resistance then have to be controlled for infection by parasites and especially the very commonly found *Wolbachia*. These bacteria have been detected in most insect orders, infecting at least 15% of all insect species worldwide, and they are believed to be a pervasive endosymbiont (Werren and Windsor, 2000), particularly in mosquito species (Kittayapong et al., 2000). Studies of life-history traits in relation to insecticide resistance must take into account the presence of *Wolbachia*, but also of other parasites, including those of medical importance. For example, insecticide resistance has been selected in field populations of several malaria vectors, which could then directly affect the transmission of malaria with deep consequences on their epidemiology (cf. McCarroll et al., 2000). Polymorphisms in traits affecting outcomes of symbiotic interactions may be maintained through selection pressures from environment, as exemplified by insecticides. The understanding of how distributed outcomes of symbiosis between populations and how outcomes evolve now need to develop an integrative approach, taking into account selection from environment.

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*Capsule-transmitted obligate gut
bacterium of plataspid stinkbugs:
a novel model system for
insect symbiosis studies*

Takema Fukatsu and Takahiro Hosokawa

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Introduction

Symbiotic microorganisms are universally found in the gut, body cavity, or cells of a wide variety of insects. Some obligate symbionts are of a mutualistic nature and contribute to the fitness of their hosts, whereas other facultative symbionts are rather parasitic and tend to cause negative effects on their hosts (Bourtzis and Miller, 2003, 2006).

In particular, the most intimate mutualistic associations are found in obligate endocellular symbionts like *Buchnera* in aphids and *Wigglesworthia* in tsetse flies. In these insects, the symbiotic bacteria are housed in the cytoplasm of large specialized cells called bacteriocytes (or mycetocytes). In the body of the insects, these cells aggregate into a large symbiotic organ called bacteriome (or mycetome), wherein the inhabiting symbionts play their physiological roles such as provisioning of essential nutrients for the host insects (Douglas, 1998; Baumann et al., 2000; Shigenobu et al., 2000; Akman et al., 2002).

Meanwhile, facultative endosymbionts such as *Wolbachia* in diverse insects and *Spiroplasma* in fruit flies are generally not restricted to specialized cells in the host body, are not essential for survival and reproduction of their hosts, and are almost neutral or rather negative to the host fitness. Some of them cause reproductive aberrations of their hosts such as cytoplasmic incompatibility, male-killing, parthenogenesis, and feminization, whereby the maternally inherited endosymbionts increase their infection frequencies in the host populations often at the expense of the host fitness (O'Neill et al., 1997; Werren, 1997; Bourtzis and Miller, 2003).

Regardless of their obligate or facultative nature, these endosymbiotic bacteria are generally passed to the next host generation vertically in the maternal body at early stages of oogenesis or embryogenesis, wherein the symbiont transmission is integrated into the intricate developmental process of the host insects (Braendle et al., 2003; Veneti et al., 2004; Frydman et al., 2006). In obligate associations, neither the host nor the symbiont can survive without their partner, constituting an almost inseparable biological entity.

Among diverse insect-microbe symbioses, several model systems, such as *Buchnera* in aphids, *Wigglesworthia* in tsetse flies, *Wolbachia* in fruit flies, etc., have been investigated preferentially. These conventional models for insect symbiosis studies have their own merits and demerits. The *Buchnera*-aphid relationship has attracted much attention because of the obligate nature of the association, the endosymbiotic system with highly developed bacteriocytes, the easy rearing and handling of the insect in the laboratory, and the importance of the host insect as notorious agricultural pest. The *Wigglesworthia*-tsetse relationship has been well studied, despite the tediousness of the insect maintenance, because of the medical importance of the host insect as the vector of African sleeping disease trypanosomes. The *Wolbachia*-*Drosophila* relationship has been widely investigated because of the

sophisticated genetic and molecular tools available with the model insect, the easy rearing and handling of the insect in the laboratory, and the cytoplasmic incompatibility phenotype induced by the symbiont that is of great interest from the viewpoint of both basic and applied biology. In these conventional model systems, however, because the host and the symbiont are endocellularly integrated in an inseparable manner, experimental manipulation of the host–symbiont associations, which is essential for understanding of the functional and biological aspects of the relationships, are generally not easy to perform.

Recently, we have established and developed a novel model system, the capsule-transmitted gut symbiotic bacteria of plataspid stinkbugs, which enable unprecedented experimental, functional, and genomic approaches to the insect–bacterium mutualism. In this chapter, we review the recent advances in the studies on the plataspid symbiosis. For details, please refer to the following original literatures (Fukatsu and Hosokawa, 2002; Hosokawa et al., 2005, 2006, 2007a, 2007b, 2008).

Gut bacterial symbiosis in stinkbugs

Over 38,000 species have been described in the insect suborder Heteroptera, which are known as true bugs or stinkbugs (Schuh and Slater, 1995). In many plant-feeding stinkbugs, the terminal region of the midgut is characterized by the presence of many sacs or tubular outgrowths, called crypts or ceca, whose lumen is filled with a specific bacterial symbiont (Glasgow, 1914; Goodchild, 1963; Buchner, 1965). In some of the stinkbugs, experimental elimination of the symbiont was reported to cause retarded growth and nymphal mortality, suggesting that the symbionts play substantial biological roles for the host insects. Probably because of their extracellular associations in the gut cavity, these stinkbugs have evolved posthatch symbiont transmission mechanisms instead of the ovarian transmission mechanisms typical of the endocellular symbionts. The following mechanisms have been described thus far: superficial bacterial contamination of eggs (egg smearing) from the families Pentatomidae, Acanthosomatidae, and others (Rosenkranz, 1939; Abe et al., 1995); probing of parental bacteria-containing excrement (coprophagy) from the families Cydnidae and Coreidae (Huber-Schneider, 1957; Schorr, 1957); deposition of bacteria-containing capsules with eggs (capsule transmission) from the family Plataspidae (Schneider, 1940; Müller, 1956) (see this chapter); and acquisition of specific soil bacteria during nymphal development (environmental acquisition) from the family Alydidae (Kikuchi et al., 2007) (see Chapter 6).

Stinkbugs of the family Plataspidae

Plataspid stinkbugs are beetle-like in appearance, being ovoid or subordicular and strongly convex, with the scutellum greatly enlarged to cover almost the entire abdomen. Thus far, about 530 species and 56 genera have been described in the family Plataspidae. Some species are known as pests of crop legumes in Asia, Pacific Islands, and Australia (Schuh and Slater, 1995). From Japan, some 12 species representing three genera, *Megacopta*, *Coptosoma*, and *Brachyplatys*, have been reported (Tomokuni, 1993) (Figure 5.1).

Scientific history: discovery of symbiont capsule in plataspid stinkbug

Schneider (1940) first described the astonishingly unique formation of the symbiotic system in a European plataspid species *Coptosoma scutellatum*. Adult females of the stinkbug lay eggs in two rows on leaves or buds of the host plant (Figure 5.2A). On the underside of

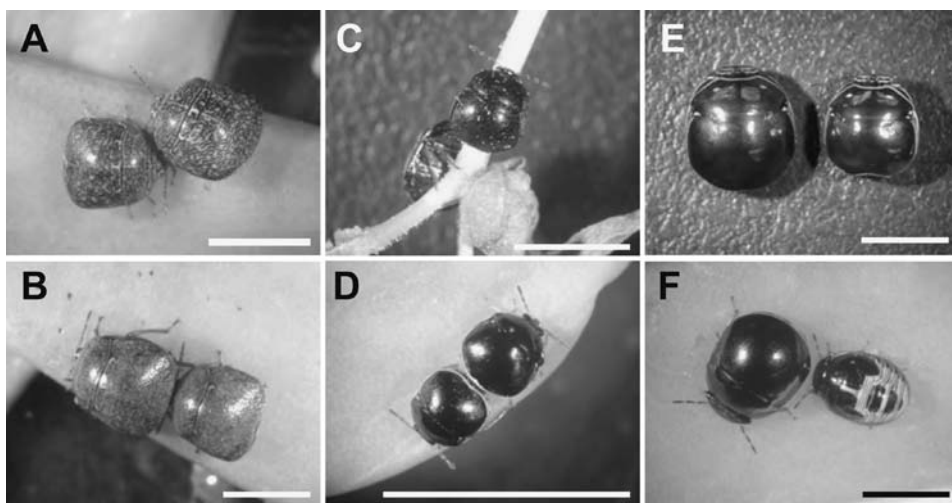


Figure 5.1 Japanese plataspid stinkbugs. (A) *Megacopta punctatissima*. (B) *M. cribraria*. (C) *Coptosoma parvipictum*. (D) *C. sphaerula*. (E) *Brachyplatys subaeneus*. (F) *B. vahllei*. Bars show 5 mm.

the egg mass, small particles, dark brown in color, are present (Figures 5.2A and B). The particles, called “symbiont capsules,” encase plenty of bacterial cells inside. Posterior mid-gut of the stinkbug is highly developed and specialized, with a number of crypts full of enormous amount of bacterial cells, being transformed into a voluminous symbiotic organ (Figure 5.2C). Namely, the symbiont capsule is a mother-made bacteria-containing “lunch box.” Newborn nymphs immediately suck the capsule content upon hatching, thereby orally acquiring the symbiont (Figure 5.2B). Later, Müller (1956) conducted some experimental studies, which demonstrated that experimental disruption of symbiont acquisition results in retarded growth and mortality of symbiont-deficient nymphs of *C. scutellatum*. Since the early pioneering works, however, nobody has worked on the intriguing subject at all.

Personal history: why has plataspid symbiosis fascinated us?

It was almost 18 years ago when I, Takema Fukatsu, first knew of the plataspid symbiosis. In the book *Endosymbiosis of Animals with Plant Microorganisms* (Buchner, 1965), I encountered an arresting illustration of the stinkbug nymph probing the symbiont capsule (cf. Figure 5.2B). Scrutinizing the descriptions on the issue, I immediately understood what an unprecedented and promising system the plataspid stinkbug offers for experimental, functional, and evolutionary studies on insect–bacterium mutualism. I was fascinated by the “symbiont capsule,” and truly would have liked to work on it. At that time, however, I was in the first year of my graduate course at the University of Tokyo, just starting a project on the diversity and evolution of aphid endosymbiotic systems under the supervision of Prof. Hajime Ishikawa. On account of no more time and resources for another project, I had to give up the idea to work on the fantastic system. However, I envisioned that “In future, when the time comes, if nobody will have not yet worked on the symbiotic system, I will do study it.” Because the plataspid symbiosis seemed so exciting to me, I thought that somebody else would sooner or later start working on it. Unexpectedly and fortunately,

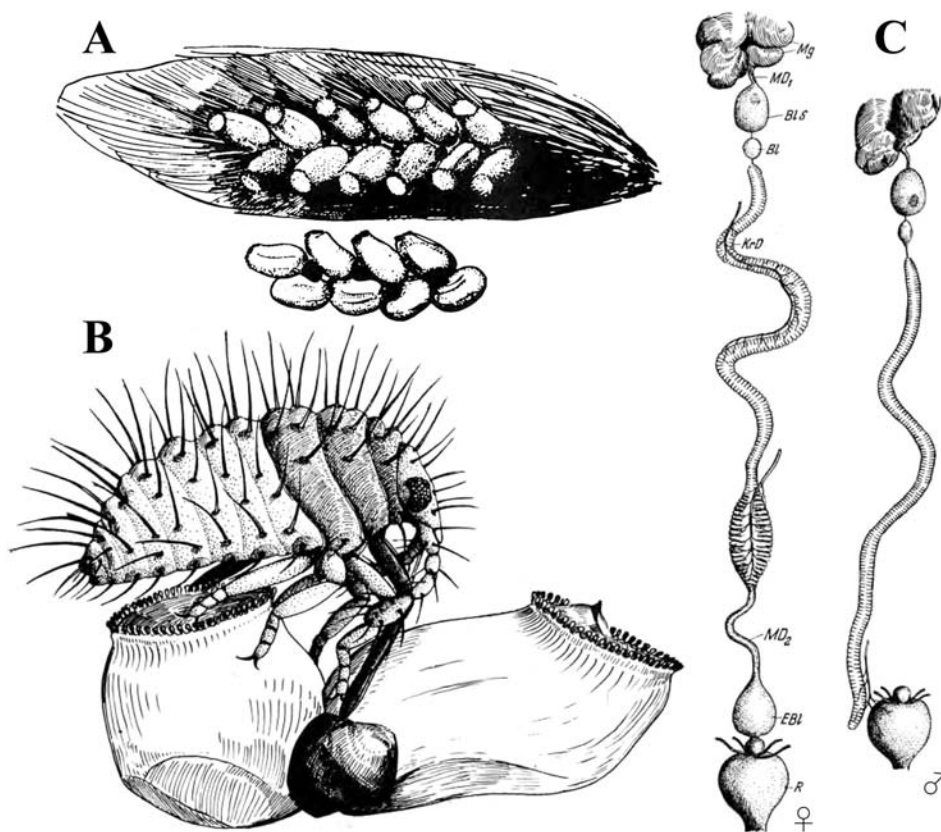


Figure 5.2 Early descriptions of the symbiotic system in the European plataspid stinkbug *Coptosoma scutellatum*. (A) Upper view (top) and lower view (bottom) of egg masses (Schneider, 1940). (B) A newborn nymph probing a symbiont capsule (Müller, 1956). (C) Alimentary tracts of adult female (left) and male (right) (Schneider, 1940).

however, nobody has paid attention to the phenomenon at all. Since then, time has passed, and in 2001, when I had already organized my own research group, I got acquainted with an indefatigable graduate student, Takahiro Hosokawa, who was working on mating behavior of the plataspid stinkbug *Megacopta punctatissima* and knew everything about the insect biology and ecology. I persuaded him by saying, “Not only insects but all animals mate. Working on mating behavior cannot be special, rather boring. But see the capsule-mediated symbiont transmission in the stinkbug! No other organisms have such a unique system. You are able to develop highly original works with it!” In this way, our fruitful collaboration started.

Symbiotic system of the Japanese common plataspid stinkbug Megacopta punctatissima

The plataspid stinkbug, *Megacopta punctatissima* (Figure 5.1A), is commonly found everywhere in mainland Japan, feeding mainly on the leguminous vine, *Pueraria lobata*, although

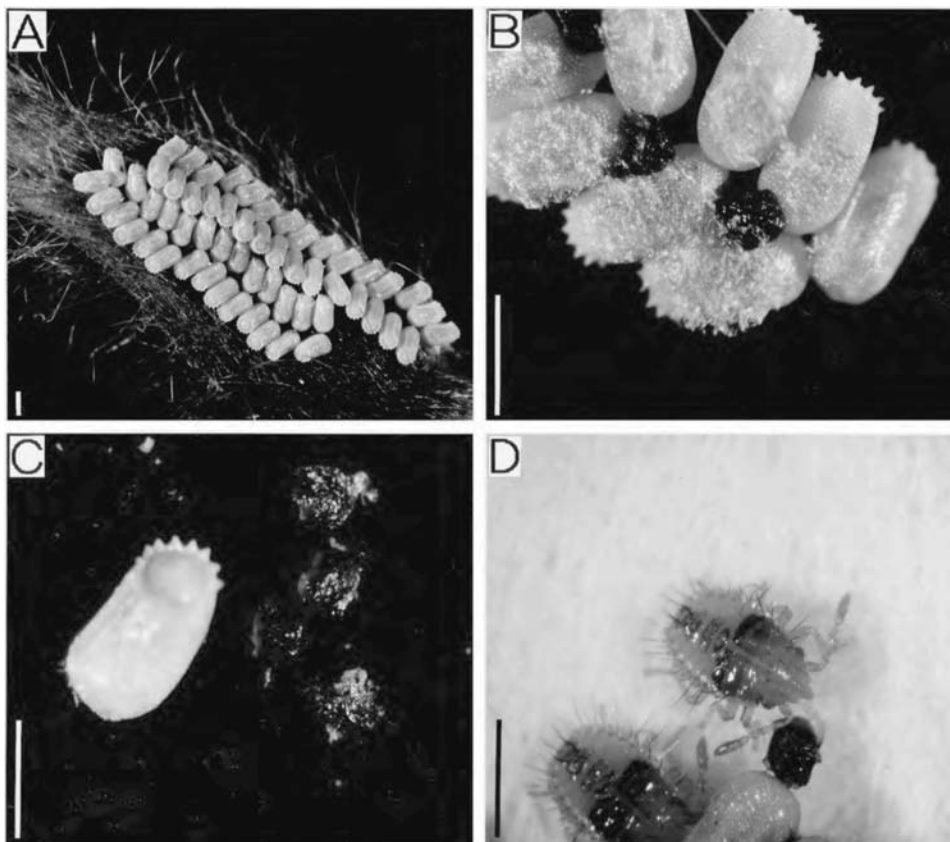


Figure 5.3 (Color figure follows p. 238.) (A) Egg masses of *Megacopta punctatissima* laid on a bud of *Pueraria lobata*. (B) Symbiont capsules, dark brown in color, placed on the underside of an egg mass. (C) An isolated egg and capsules. (D) A newborn nymph probing a symbiont capsule. Bars show 0.5 mm. (From Fukatsu, T., and Hosokawa, T. [2002]. *Appl. Environ. Microbiol.* **68**: 389–396. With permission.)

other leguminous plants, including crop legumes like soybean and pea, are occasionally utilized (Tomokuni, 1993). From May to July, adult females lay egg masses on buds of *P. lobata*. An egg mass consists of 5 to 51 eggs arranged in two rows in parallel (Figure 5.3A). On the underside of the egg masses, symbiont capsules, 0.2 to 0.3 mm in diameter and dark brown in color, are always attached (Figure 5.3B). The aubergine-shaped eggs and the capsules are easily separable with forceps under a dissection microscope (Figure 5.3C). Within a week after oviposition, first instar nymphs emerge from the eggs, and the newborn nymphs immediately probe the capsules with their proboscis for 1 hour or so (Figure 5.3D). After sucking the capsule, they enter a resting period in aggregation nearby the eggshells for 1 or 2 days, and then disperse for feeding on the plant sap.

Figure 5.4 shows the internal structure of the symbiont capsule. Light microscopy indicated that the capsule consists of two distinct components, namely, a layered envelope and the capsule content (Figure 5.4A). Careful dissection of fresh capsules revealed the cuticle-like envelope and the resin-like content, both of which are brownish in color. Electron microscopy unveiled that the envelope encases not only bacterial cells but also a matrix

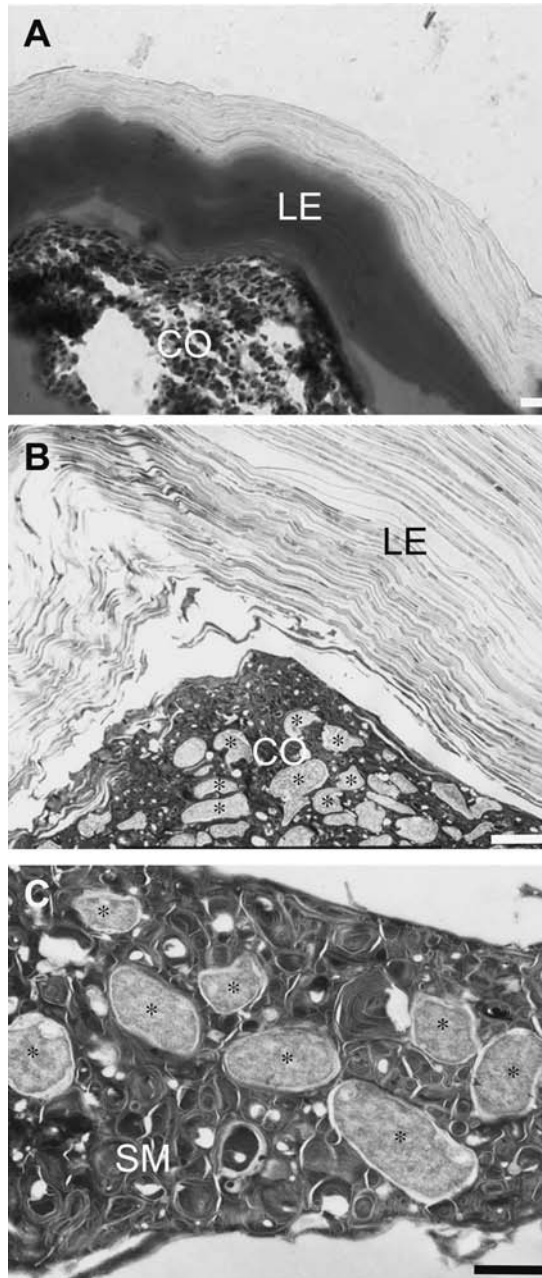


Figure 5.4 (Color figure follows p. 238.) Light and electron microscopy of sectioned symbiont capsules of *Megacopta punctatissima*. (A) Light microscopic image of a symbiont capsule. (B) Electron microscopic image of a symbiont capsule. Symbiont cells are shown by asterisks. (C) Electron microscopic image of the capsule content. Abbreviations: CO, capsule content; LE, layered envelope; SM, secretion matrix. Bars show 2 μm in (A) and (B), and 1 μm in (C). (From Hosokawa, T., Kikuchi, Y., Meng, X.Y., and Fukatsu, T. [2005]. *FEMS Microbiol. Ecol.* 54: 471–477. With permission.)

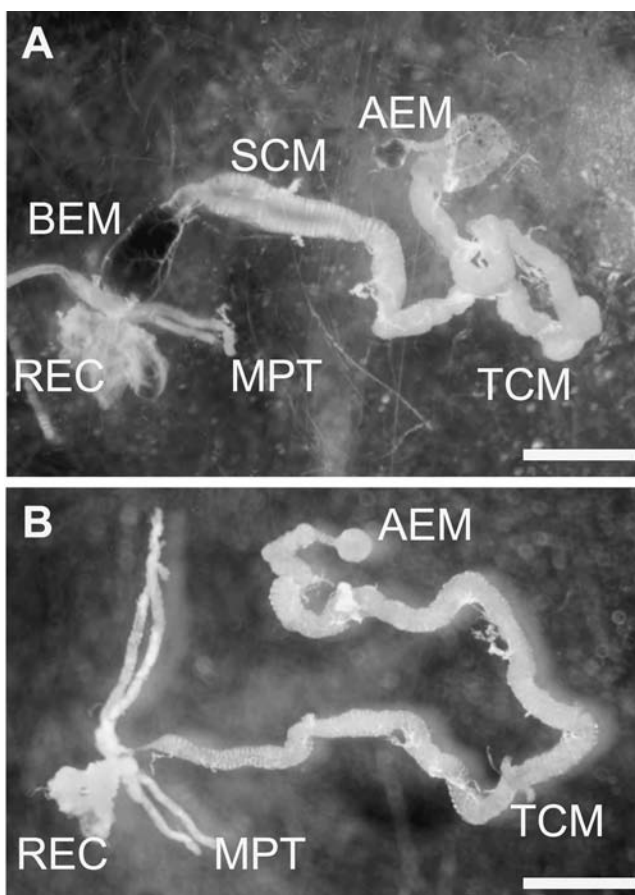


Figure 5.5 (Color figure follows p. 238.) Dissected posterior midgut from adult female (A) and adult male (B) of *Megacopta punctatissima*. Abbreviations: AEM, anterior enlarged midgut section; BEM, brownish enlarged midgut end section; MPT, Malpighian tubules; REC, rectum; SCM, swollen crypt-bearing midgut section; TCM, thin crypt-bearing midgut section. Bars show 1 mm. (From Hosokawa, T., Kikuchi, Y., Meng, X.Y., and Fukatsu T. [2005]. *FEMS Microbiol. Ecol.* **54**: 471–477. With permission.)

(Figures 5.4B and C). The symbiont cells were buried in the matrix rather sparsely, accounting for less than half of the volume of the capsule content (Figure 5.4C).

Figure 5.5 shows the posterior midgut dissected from adult insects of *M. punctatissima*. In females, anatomically distinct sections were recognized along the alimentary tract: an anterior enlarged midgut (AEM) section, a thin crypt-bearing midgut (TCM) section, a swollen crypt-bearing midgut (SCM) section, and a brownish enlarged midgut (BEM) end section (Figure 5.5A). In males, no such specializations were found: nearly the full length of the alimentary tract was represented by a TCM section while SCM and BEM sections were lacking (Figure 5.5B).

Figure 5.6 shows the light and electron microscopy of the female alimentary tract, which unveiled functional specialization of each of the midgut sections. The TCM section, accounting for the largest part of the posterior midgut, bore a number of voluminous crypts (Figure 5.6A). The crypts were lined with very thin epithelial cells and filled with

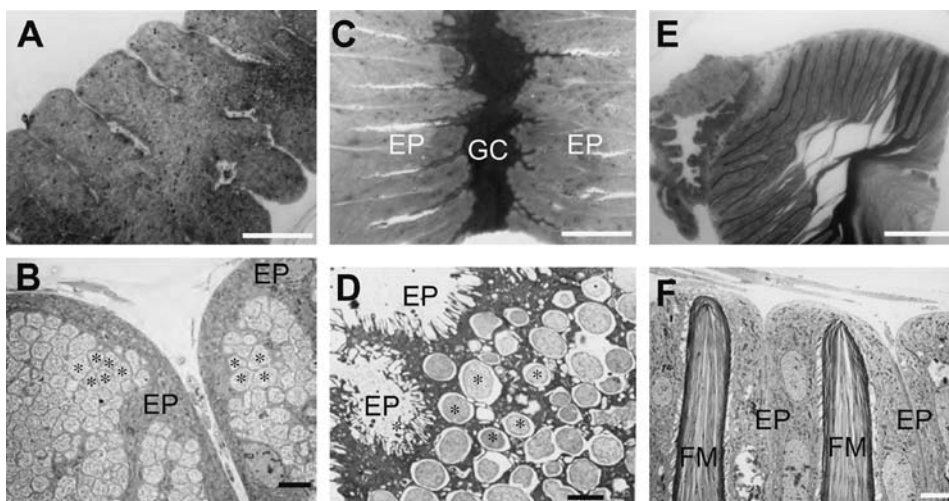


Figure 5.6 (Color figure follows p. 238.) Light and electron microscopy of the midgut sections of *Megacopta punctatissima*. (A) Thin crypt-bearing midgut section (TCM). (B) Crypts of TCM, where numerous symbiont cells (asterisks) and thin epithelium are seen. (C) Swollen crypt-bearing midgut section (SCM). (D) Crypt of SCM, where the matrix is secreted. In the main tract of the midgut, a number of symbiont cells (asterisks) are embedded in the matrix. (E) Brownish enlarged midgut end section (BEM). (F) Crypts of BEM, whose cavity is filled with filament-like materials of the capsule envelope. Abbreviations: EP, epithelium; FM, filament-like material; GC, gut content. Bars show 50 μ m in (A), (C), and (E), and 2 μ m in (B), (D), and (F). (From Hosokawa, T., Kikuchi, Y., Meng, X.Y., and Fukatsu T. [2005]. *FEMS Microbiol. Ecol.* 54: 471–477. With permission.)

symbiotic bacteria. The symbionts were tightly packed in the cavity of the crypts, and accounted for most of the volume of the gut content (Figure 5.6B). The TCM section is the place for harboring the symbiotic bacteria. The SCM section bore a number of crypts with thick epithelia, and most of the gut content was present not in the crypts but in the main tract of the midgut section (Figure 5.6C). The glandular epithelial cells were rich in microvilli and secretion granules containing electron-dense materials. The materials were secreted into the gut cavity, forming a secretion matrix embedding the symbiont cells (Figure 5.6D). Hence, the SCM section is the organ specialized for production of the matrix. The BEM section consisted of deep and narrow crypts, whose cavity was filled with filament-like materials (Figure 5.6E). The fine structure of the materials (Figure 5.6F) was identical to that of the envelope of the symbiont capsule (Figure 5.4B). Hence, the BEM section is the organ specialized for production of the cuticular envelope materials.

Posterior midgut of plataspid stinkbug as specialized symbiotic organ

Anatomically, plataspid stinkbugs are very unique in that their alimentary tract is completely disconnected in the midway. In newborn nymphs, their gut is normally organized, which allows the ingested symbiont to colonize the midgut. In the developmental course, however, the midgut is constricted into anterior and posterior parts. In adult insects, the anterior midgut is free of the symbiont, being connected to the posterior midgut only with a delicate membranous thread without cavity (cf. Figure 5.2C). Judging from the peculiar anatomy, the plant sap ingested by the insect is completely absorbed in the anterior mid-

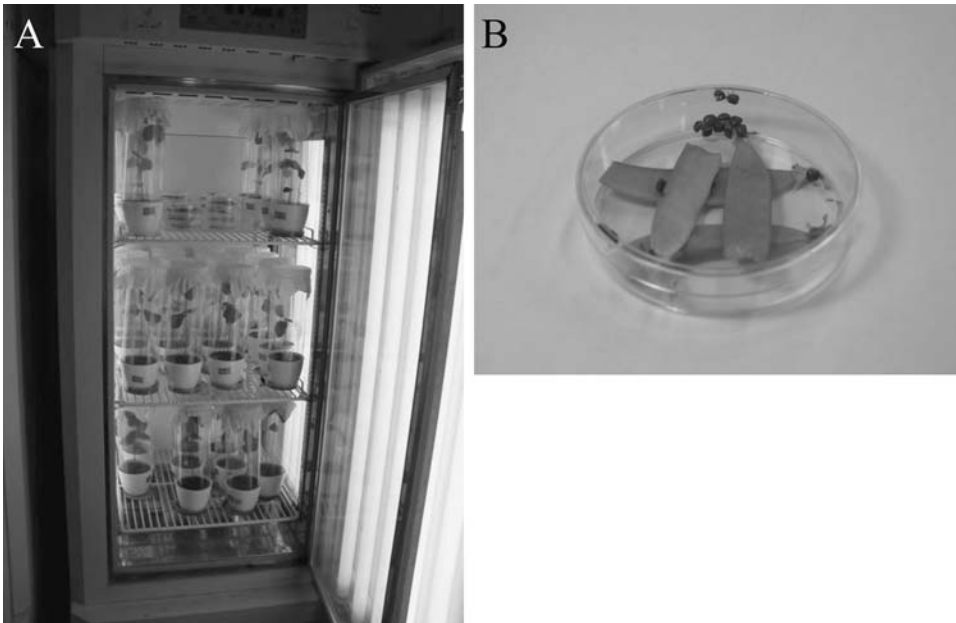


Figure 5.7 Rearing systems for plataspid stinkbugs. (A) Rearing on potted soybean plants. (B) Petri dish rearing on pea pods.

gut, the waste is excreted through the Malpighian tubules into the hindgut, and there is no food flow through the posterior midgut. In this way, the posterior midgut is transformed into a voluminous organ for harboring a huge amount of the symbiont in the cavity (cf. Figures 5.2, 5.5, and 5.8). Hence, the posterior midgut of plataspid stinkbugs can be regarded as “pseudo-bacteriome” in that the symbiotic bacteria are not harbored in the cytoplasm but in the extracellular cavity.

Laboratory rearing of plataspid stinkbugs

For detailed experimental and biological studies, the insect materials of interest must be constantly maintained and supplied in the laboratory. For that purpose, we established several rearing systems for Japanese plataspid stinkbugs, whereby the insects were stably maintained and bred at least for several generations. On potted plants of the soybean (*Glycine max*), nymphs and adults of many species including *M. punctatissima*, *M. cribraria*, *C. sphaerula*, *B. subaeneus*, and *B. vahlii* were stably maintainable (Figure 5.7A). Potted plants of buckwheat (*Fagopyrum esculentum*) were suitable for rearing of *C. parvipictum*. For mating adult insects and harvesting egg masses with symbiont capsules, Petri dish rearing with pea pods (*Pisum sativum*) worked quite well and allowed easy experimental handling (Figure 5.7B). These insects were maintained at 25°C under a long day regimen (16L:8D).

Prevalence of maternal capsule production in plataspid stinkbugs

We collected plataspid stinkbugs from all over Japan, and examined *M. punctatissima*, *M. cribraria*, *B. subaeneus*, *B. vahlii*, *C. parvipictum*, *C. sphaerula*, and *C. japonicum* for their

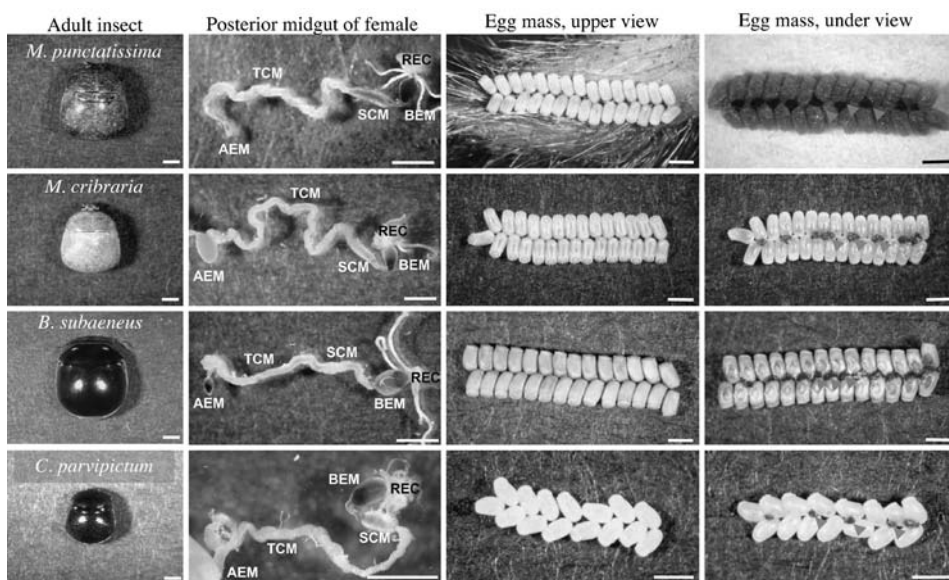


Figure 5.8 (Color figure follows p. 238.) Four representative species of Japanese plataspid stinkbugs, their posterior midgut with capsule-producing organs, and their egg masses with symbiont capsules.

symbiotic system and capsule production. In all the plataspid species, the anatomical traits specialized for capsule production in the posterior midgut of female insects and the capsule production in association with oviposition were consistently observed (Figure 5.8), indicating that the capsule-mediated symbiont transmission is highly conserved among the diverse plataspid stinkbugs.

Phylogenetic placement of plataspid symbionts

A 1.5 kb bacterial 16S rRNA gene segment was amplified by PCR, cloned, and sequenced from the posterior midgut and the capsules of the plataspid stinkbugs. Each of the insect species contained a single type of the nucleotide sequence, indicating that a single and specific bacterium is associated with each of the plataspid stinkbugs. Molecular phylogenetic analyses revealed that the sequences formed a highly supported monophyletic group in the γ -Proteobacteria. The sister group of the plataspid symbionts was identified to be *Buchnera aphidicola*, the obligate endocellular symbionts of aphids (Figure 5.9). These results indicated that the capsule-transmitted gut symbionts of the plataspid stinkbugs comprise a distinct and coherent bacterial group in the γ -Proteobacteria.

Production of symbiont-free insects by removal of capsules

Four plataspid species, *M. punctatissima*, *M. cribraria*, *B. subaeneus*, and *C. parvipictum*, which were stably maintainable in the laboratory, were subjected to the following experimental analyses. Each of the egg masses of the plataspid stinkbugs was divided into two portions. One of the halves was left untreated, the other of the halves was deprived of all capsules, and newborn nymphs from these experimental egg masses were subjected to

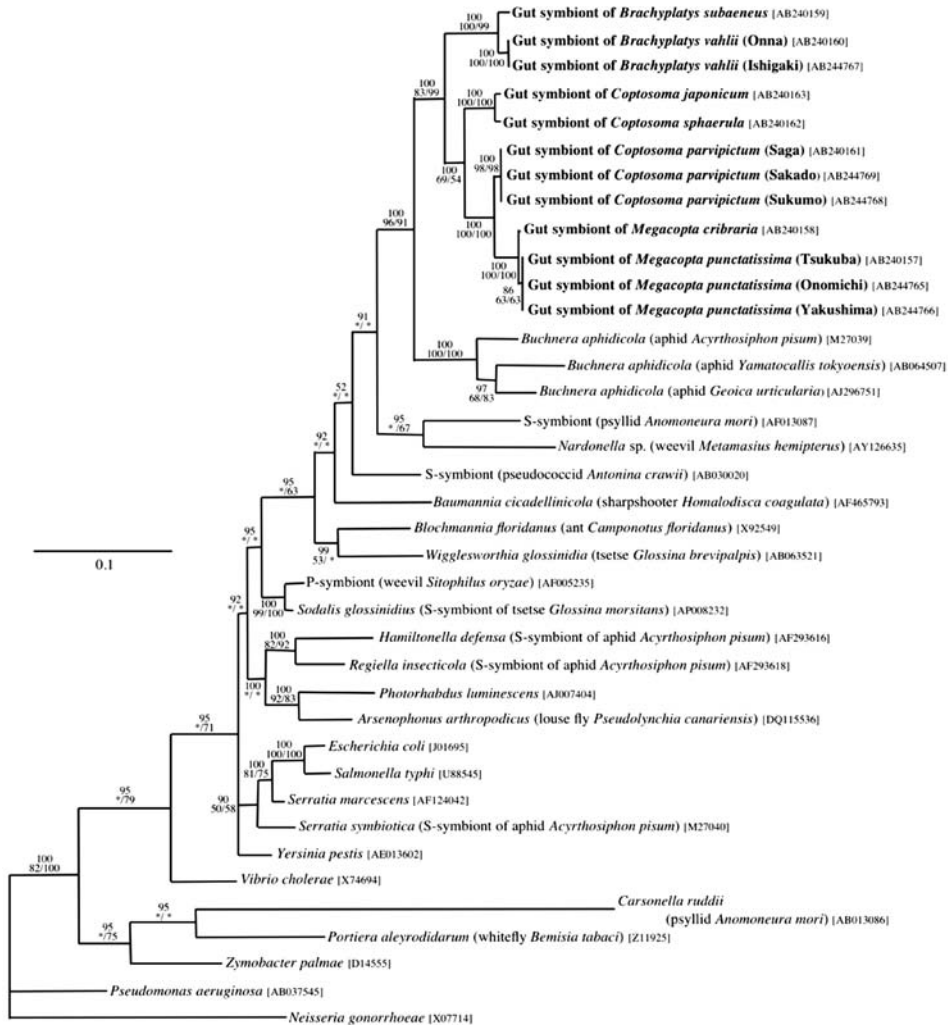


Figure 5.9 Phylogenetic placement of the symbiotic bacteria from the plataspid stinkbugs in the γ -subclass of the *Proteobacteria* on the basis of 16S rRNA gene sequences. (From Hosokawa, T., Kikuchi, Y., Nikoh, N., Shimada, M., and Fukatsu T. [2006]. *PLoS Biol.* 4: e337. With permission.)

PCR detection of the symbiont after 1 day of hatching. Almost all the nymphs from the control egg masses with capsules possessed the symbiont, whereas most of the nymphs from the treated egg masses without capsules failed to acquire the symbiont. In this way, we successfully obtained sibling populations of symbiotic and aposymbiotic insects in the four plataspid representatives.

Effects of symbiont infection on host fitness and phenotype

In all of the four species, the adult emergence rate without symbiont capsules was drastically reduced in comparison to that with symbiont capsules. In *M. punctatissima* and *M.*

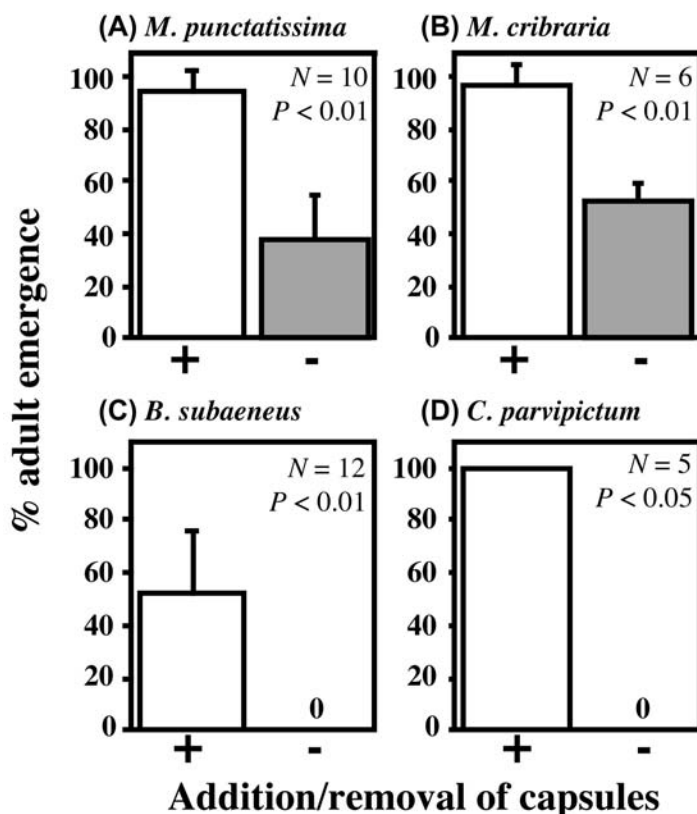


Figure 5.10 Effects of symbiont elimination on adult emergence rate of the plataspid stinkbugs. (A) *Megacopta punctatissima*. (B) *Megacopta cribraria*. (C) *Brachyplatys subaeneus*. (D) *Coptosoma parvipictum*. (From Hosokawa, T., Kikuchi, Y., Nikoh, N., Shimada, M., and Fukatsu T. [2006]. *PLoS Biol.* 4: e337. With permission.)

cribraria, about 50% of aposymbiotic nymphs died during developmental course (Figures 5.10A and B). In *B. subaeneus* and *C. parvipictum*, all of the aposymbiotic nymphs died before adult emergence (Figures 5.10C and D). In *M. punctatissima* and *M. cribraria*, about half of the aposymbiotic nymphs managed to become adults, but they exhibited abnormal phenotypes such as pale coloration, small body size, etc. (Figure 5.11). These aposymbiotic adult insects neither copulated nor reproduced.

Essential role of gut symbionts for host stinkbug

From these results, it was concluded that the capsule-transmitted gut symbiotic bacteria are essential for normal development and reproduction of the plataspid stinkbugs. The gut symbiotic bacteria can be regarded as obligate mutualistic associates for the plataspid stinkbugs, as the endocellular symbiotic bacteria *Buchnera* for the host aphids. Although it is unknown how the gut symbiotic bacteria support the growth and reproduction of the host stinkbugs, probably the symbiont provides the host with nutritional supplements,

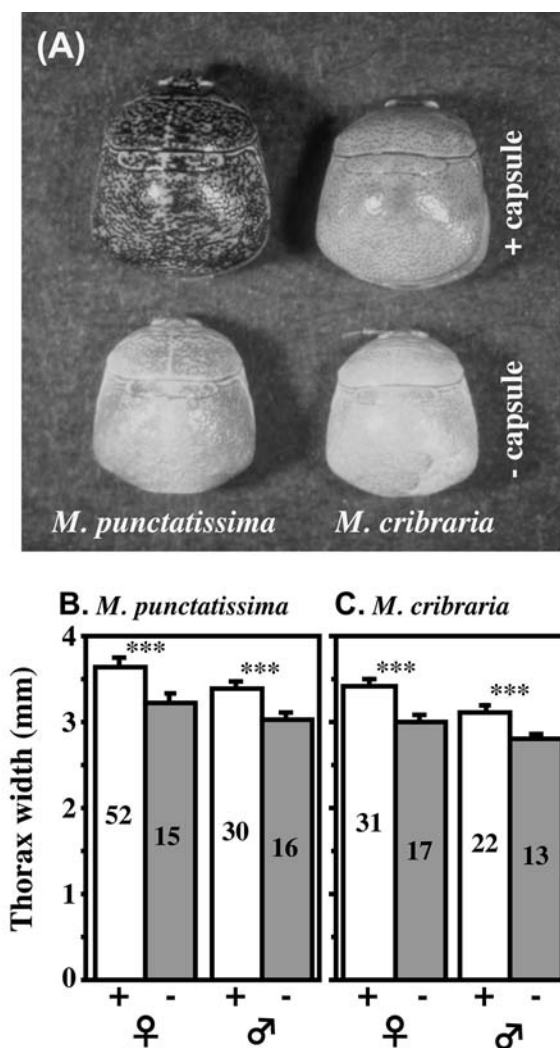


Figure 5.11 Effects of symbiont elimination on adult body size and phenotype of the plataspid stinkbugs. (A) Adult females of *M. punctatissima* (left) and *M. cribraria* (right) emerged from the control egg masses with capsules (top) and those from the treated egg masses without capsules (bottom). (B) Thorax width of *M. punctatissima* and (C) thorax width of *M. cribraria*. (From Hosokawa, T., Kikuchi, Y., Nikoh, N., Shimada, M., and Fukatsu T. [2006]. *PLoS Biol.* 4: e337. With permission.)

such as essential amino acids and vitamins, as has been reported for other plant-sucking insects (Douglas et al., 1998; Baumann et al., 2000; Shigenobu et al. 2000).

Host–symbiont cospeciation despite the midgut extracellular symbiosis

Figure 5.12 shows the comparison between the host phylogeny and the symbiont phylogeny of the plataspid stinkbugs. Strikingly, the phylogenetic relationship of the host insects agrees perfectly with the phylogenetic relationship of their symbiotic bacteria. On account

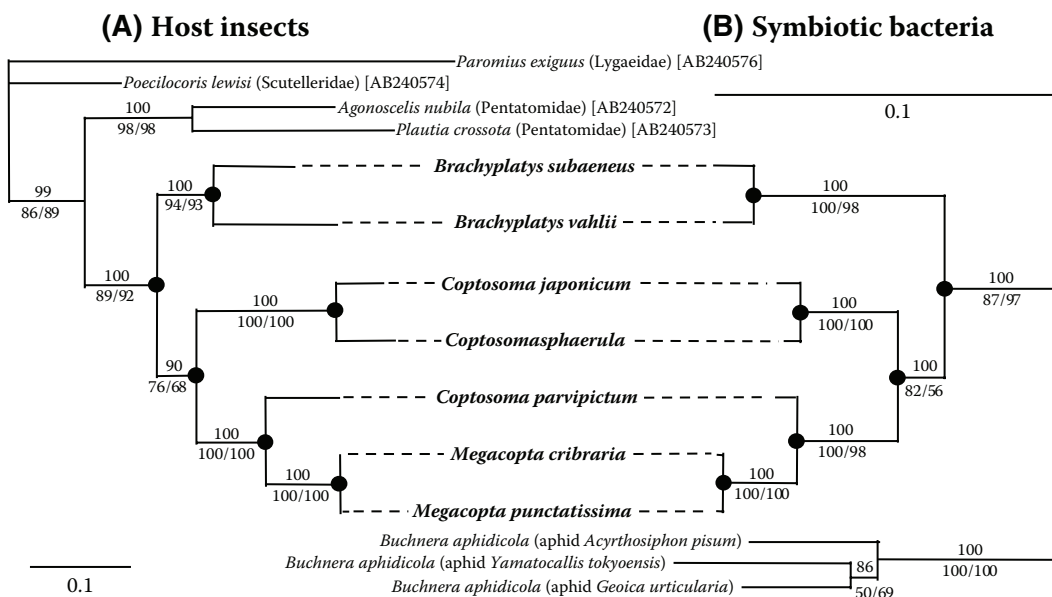


Figure 5.12 Phylogenetic congruence between the plataspid stinkbugs and their symbiotic bacteria. (A) The host insect phylogeny on the basis of mitochondrial 16S rRNA gene sequences. (B) The symbiont phylogeny on the basis of bacterial 16S rRNA gene sequences. (From Hosokawa, T., Kikuchi, Y., Nikoh, N., Shimada, M., and Fukatsu T. [2006]. *PLoS Biol.* **4**: e337. With permission.)

of a total of 10,395 possible rooted tree topologies for seven taxa, the chance that the symbiont tree will exactly match the host tree is expected to be less than 0.01%. These results indicate that the evolutionary history of the gut symbiotic bacteria mirrors that of their host plataspid stinkbugs; the pattern so-called cospeciation or cocoladogenesis. Similar cocoladogenetic patterns have been reported from diverse insect taxa associated with obligate endocellular bacteria (Chen et al., 1999; Clark et al., 2000; Sauer et al., 2000; Thao et al., 2000, 2002; Thao and Baumann 2004; Moran et al., 2003; Lefevre et al., 2004). To our knowledge, this study is the first to identify a strict cocoladogenesis between a group of insects and their gut symbionts. The phylogenetic congruence strongly suggests that a single bacterial infection in the common ancestor of the plataspid stinkbugs has been stably maintained over evolutionary time without effective horizontal transfers and has been diversified in parallel with the host speciation.

General patterns in the genome evolution of endocellular symbiotic bacteria of insects

Recent molecular evolutionary analyses have suggested that the endocellular lifestyle of obligate insect symbionts has strongly affected their genome evolution, causing AT-biased nucleotide composition, accelerated rate of molecular evolution, and significant genome size reduction (Mira et al., 2001; Wernegreen, 2002). These peculiar genetic traits are hypothesized to be the consequence of attenuated purifying selection due to small population size and strong bottleneck, which are associated with the lifestyle of vertically transmitted endocellular symbionts (Moran, 1996; Wernegreen, 2002). Here it should

be noted that small population size and strong bottleneck are also found in vertically transmitted extracellular symbionts like those of the plataspid stinkbugs. Thus, molecular evolutionary analyses of the plataspid symbionts will provide an opportunity to identify the principal factor responsible for the reductive genome evolution. If the population's genetic attributes such as small population size and bottleneck have the principal effect, the extracellular symbionts will also exhibit the peculiar genetic traits. If the endocellular environment itself is the principal factor, the peculiar genetic traits will be less conspicuous or absent in the extracellular symbionts.

Reductive genome evolution in the midgut extracellular symbiotic bacteria

Interestingly, the gut symbiotic bacteria of the plataspid stinkbugs exhibited those peculiar genetic traits. The nucleotide compositions of their 16S rRNA gene sequences were AT-biased, ranging from 50% to 54%, which are remarkably higher than the values of free-living γ -Proteobacteria (around 45%) and equivalent to the values of obligate endocellular γ -Proteobacterial symbionts of other insects (50%–64%). The molecular evolutionary rates of their 16S rRNA gene sequences were significantly accelerated: about 6.3 times higher than the rates in related free-living γ -Proteobacteria and almost equivalent to the rates in the lineage of the aphid endocellular symbionts *Buchnera*. Pulsed field gel electrophoresis revealed that their genomes were drastically reduced to be around 0.8 Mb in size (Figure 5.13). The genome size was significantly smaller than those of free-living γ -Proteobacteria such as *Escherichia coli* (4.6 Mb) (Blattner et al., 1997), *Vibrio cholerae* (4.0 Mb) (Heidelberg et al., 2000), and *Pseudomonas aeruginosa* (6.3 Mb) (Stover et al., 2000), and were almost equivalent to those of endocellular symbionts such as *Buchnera* of aphids (0.45–0.65 Mb) (van Ham et al., 2003), *Wigglesworthia* of tsetse flies (0.70 Mb) (Akman et al., 2002), *Blochmannia* of ants (0.81 Mb) (Gil et al., 2003), and *Baumannia* of sharpshooters (0.69 Mb) (Wu et al., 2006). These results strongly favor the hypothesis that attenuated purifying selection due to small population size and strong bottleneck is the principal factor that has shaped the peculiar genetic traits of the obligate insect symbionts in general.

Proposal of “Candidatus Ishikawaella capsulata”

On account of the phylogenetically and biologically distinct traits as described above, the designation “*Candidatus Ishikawaella capsulata*” was proposed for the symbiotic bacteria of the plataspid stinkbugs (Hosokawa et al., 2006). The generic name honors Prof. Hajime Ishikawa, who pioneered molecular biological studies on insect symbiosis and passed away recently (Fukatsu, 2006). The specific name indicates the “capsule” encasing the symbiont.

Plataspid–Ishikawaella symbiosis: a novel model system for insect symbiosis studies

The unique symbiotic system of the plataspid stinkbugs, wherein host eggs and symbiont capsules are separable by using forceps under binocular microscope, enables novel experimental approaches to previously untouched aspects of the insect–microbe mutualism. The unprecedented uniqueness of the system resides in the following points:

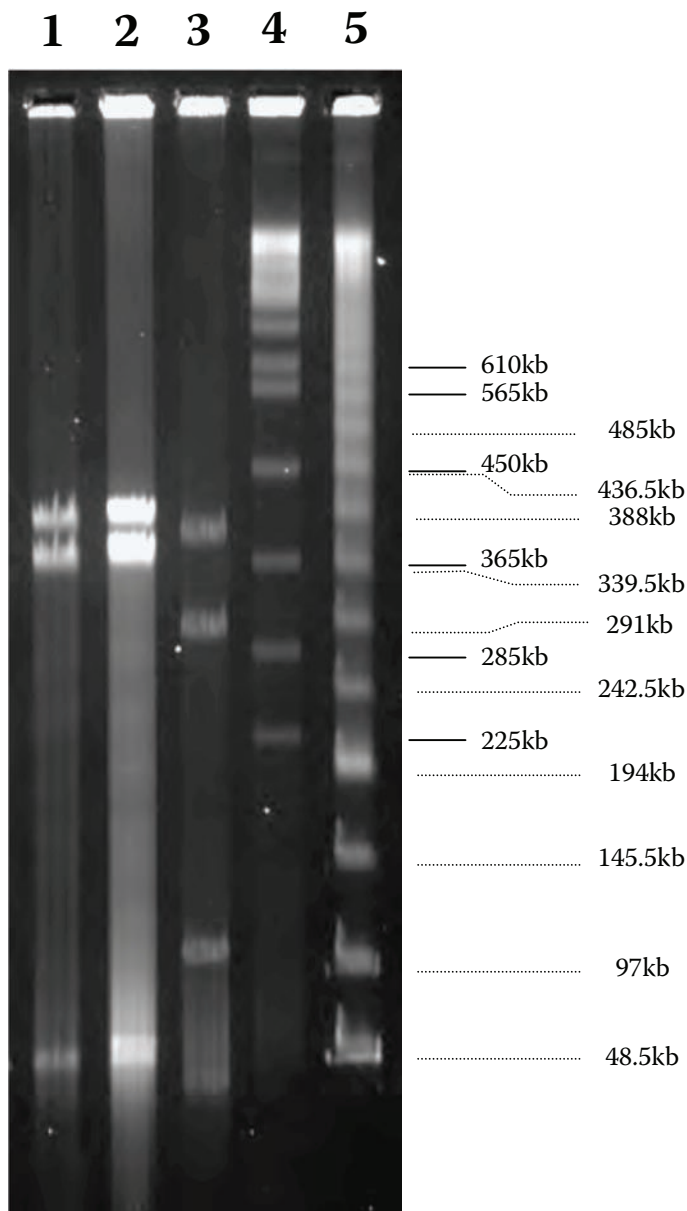


Figure 5.13 Pulsed field gel electrophoresis of the symbiont genomic DNA prepared from posterior midgut of an adult female of the plataspid stinkbugs. Lane 1, *M. punctatissima*; lane 2, *M. cribraria*; lane 3, *C. parvipictum*; lane 4, yeast PFGE marker; lane 5, lambda PFGE marker. Marker sizes are shown on the right side. (From Hosokawa, T., Kikuchi, Y., Nikoh, N., Shimada, M., and Fukatsu T. [2006]. *PLoS Biol.* 4: e337. With permission.)

1. The symbionts are not directly passed to the eggs but packaged in the capsules.
2. Hence, the host and the symbiont are easily separable and manipulatable.
3. Vertical transmission process of the symbiont is observed as nymphal behavior, and thus manipulatable.

4. The maternal investment in the symbiont transmission is measurable in terms of capsule production, symbiont titer, etc.
5. Although essential for their host insects, the symbionts are not endocellular but located in the midgut cavity extracellularly.
6. Many host species possess the same symbiotic system, providing an excellent experimental model for studying coevolutionary aspects.
7. By exchanging symbiont capsules between egg masses, host-symbiont associations can be experimentally manipulated between populations, species, and genera of the host insects.
8. Preparation of the symbiont of high purity and quantity is easy, which is suitable for biochemical and genomic analyses.

Hereafter, we briefly introduce two lines of our recent works (Hosokawa et al., 2007b; 2008) that unveiled intriguing aspects of host-symbiont interactions by making full use of the unique symbiotic system of the plataspid stinkbugs.

Symbiont acquisition alters behavior of nymphal stinkbugs

Effects of parasitic symbiont on host behavior and biology

In parasitic associations, the evolutionary interest of the symbiont contradicts that of the host, which sometimes causes the phenomena so-called “manipulation of host behavior by parasite” or simply “parasite manipulation” (Moore, 2002; Thomas et al., 2005). For example, the behavior of parasitoid wasp *Leptopilina boulardi* is affected by a virus to increase superparasitism, whereby horizontal transmission of the virus is significantly enhanced at the expense of reproductive success of the host (Varaldi et al., 2003). Meanwhile, the targets of parasite manipulation are not only restricted to behavioral traits but can also be morphological, physiological, or reproductive ones. For example, endocellular bacteria of the genus *Wolbachia* cause reproductive aberrations of the host arthropods, by which symbiont vertical transmission is facilitated at the expense of host fitness (O'Neill et al., 1997; Werren, 1997; Bourtzis and Miller, 2003).

Effects of mutualistic symbiont on host behavior and biology

In mutualistic associations, by contrast, the evolutionary interest of the symbiont parallels that of the host. Fidelity of transmission and stability of infection are pivotal for both of the symbiotic partners. Symbiont-induced morphogenetic, developmental, and physiological host traits that enhance transmission, stability, and functioning of the symbiont have been documented from a variety of mutualistic associations, including morphogenesis of symbiotic organs in the squid-*Vibrio* luminescent symbiosis (Nyholm and McFall-Ngai, 2004), formation of root nodules in the legume-*Rhizobium* nitrogen-fixing symbiosis (Denarie et al., 1992), and others. However, symbiont-induced alteration of the host behavior that enhances its transmission has been, to our knowledge, scarcely described.

Resting/aggregating behavior of stinkbug nymphs

Young nymphs of stinkbugs generally exhibit characteristic resting behavior in aggregation. In the stinkbug *Nezara viridula*, the gregariousness was reported to improve developmental performance of nymphs (Kiritani, 1964; Lockwood and Story, 1986). It has been

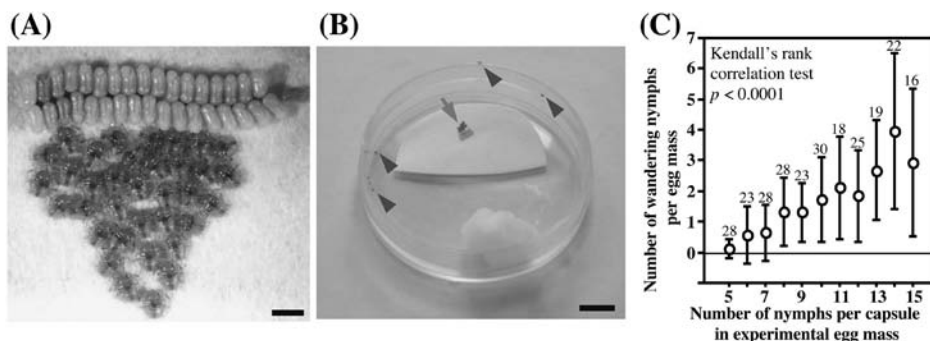


Figure 5.14 (A) Newborn nymphs of *M. punctatissima* resting in aggregation nearby the eggshells. Bar shows 1 mm. (B) Resting nymphs (arrow) and wandering nymphs (arrowheads) from an experimental egg mass that consists of 15 eggs and a capsule. Bar shows 1 cm. (C) Relationship between the number of nymphs per capsule and the number of wandering nymphs among the experimental egg masses. (From Hosokawa, T., Kikuchi, Y., Shimada, M., and Fukatsu T. [2008]. *Biol. Lett.* 4: 45–48. With permission.)

argued that the nymphal gregariousness might enhance aposematic effects against predators (Aldrich and Blum, 1978). However, actual biological significance of the resting/aggregating behavior in stinkbug nymphs has been obscure.

Nymphal resting behavior in M. punctatissima

Newborn nymphs of *M. punctatissima* immediately probed symbiont capsules with their proboscis for around 1 hour, got into a resting status nearby the eggshells for 1–2 days in aggregation (Figure 5.14A), and then dispersed to feed on plant sap. Diagnostic PCR detection confirmed that all the nymphs successfully acquired the symbiont from the capsules.

Experimental depletion of symbiont resulted in nymphal wandering behavior

By removing eggs and capsules from field-collected egg masses, we generated 260 experimental egg masses with 5–15 eggs and a single capsule, whereby the levels of symbiont supply per nymph were controlled. In these experimental egg masses, many nymphs failed to exhibit normal resting behavior, either actively wandering in the rearing container or getting immobile singly or in a group of a few insects (Figure 5.14B).

Lower symbiont supply resulted in more wandering nymphs

Figure 5.14C shows the relationship between the level of symbiont depletion and the number of wandering nymphs. In the experimental egg masses with five nymphs per capsule, few nymphs exhibited wandering behavior. However, as the number of nymphs per capsule increased, the more wandering nymphs occurred.

Wandering nymphs were certainly symbiont-depleted

Then, we generated 24 experimental egg masses consisting of 15 eggs and a capsule, and investigated the relationship between the infection status of newborn nymphs and their

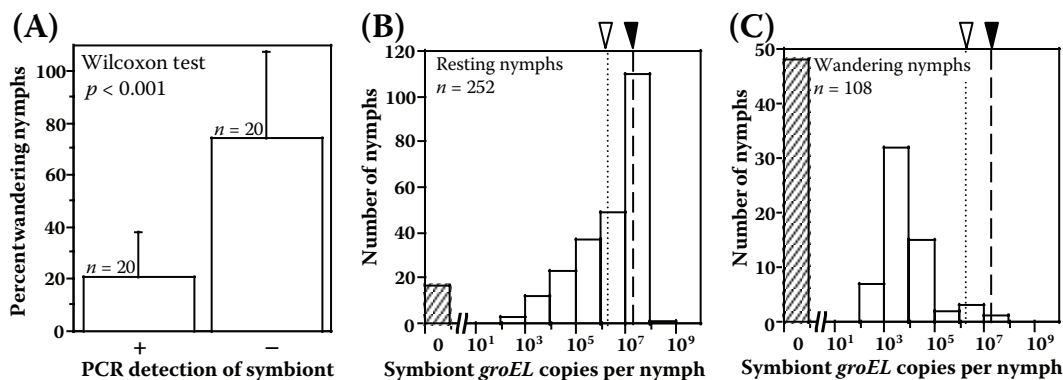


Figure 5.15 (A) Behavior of symbiont-infected and uninfected nymphs of *M. punctatissima*. (B) Frequency distribution of symbiont titers in resting nymphs. (C) Frequency distribution of symbiont titers in wandering nymphs. In (B) and (C), filled arrowheads indicate the normal symbiont titer acquired by newborn nymphs, 2×10^7 , whereas open arrowheads show the minimal symbiont titer needed for normal development of the nymphs, 2×10^6 (cf. Hosokawa et al., 2007a). (From Hosokawa, T., Kikuchi, Y., Shimada, M., and Fukatsu T. [2008]. *Biol. Lett.* 4: 45–48. With permission.)

behavior. The wandering nymphs occupied nearly 80% of the symbiont-negative insects and accounted for only 20% of symbiont-positive insects (Figure 5.15A).

Figures 5.15B and C show the distribution of symbiont titers detected in the resting nymphs and the wandering nymphs, respectively. A previous study demonstrated that nymphs of *M. punctatissima* normally acquire 2×10^7 symbionts on average, and the minimal symbiont titer needed for normal development of the nymphs is about 2×10^6 (Hosokawa et al., 2007a). Quantitative PCR assays revealed that most of the resting nymphs (93%) were infected with the symbiont, the majority of the infected nymphs exhibited symbiont titers over the threshold level of 10^6 , and the distribution peak was at the normal acquisition titer around 10^7 (Figure 5.15B). By contrast, only 55.6% of the wandering nymphs were infected with the symbiont and most of the infected nymphs exhibited symbiont titers below 10^6 (Figure 5.15C).

Insights into behavioral biology of symbiont-associated insects

On the basis of these results, we conclude that, in *M. punctatissima*, sufficient symbiont acquisition induces nymphal resting behavior in aggregation while insufficient symbiont acquisition results in nymphal wandering behavior. It is expected, although speculative, that the behavioral patterns of the nymphs ensures transmission of the essential symbiont and minimizes the energy and time spent for the activity.

In the case of *M. punctatissima*, the behavioral alteration associated with the symbiont acquisition may be an adaptive behavioral response of the host insect rather than a consequence of behavioral manipulation by the bacterial symbiont. However, because the host and the symbiont both benefit from the behavior, the behavior might have been evolutionarily favored by selection pressures acting on both the partners.

Resting behavior of newborn nymphs in aggregation is generally found among diverse stinkbugs, but biological significance of the behavior has been poorly understood. Our finding suggests the possibility that, although speculative, the resting behavior might be

involved in the initial establishment of the symbiosis in the midgut of this and other stinkbug species.

This study highlights the possibility that specialized behavioral traits contribute to the maintenance of mutualistic host–symbiont associations, and thus can evolve for that purpose. We point out that, in addition to the well-known cases of parasite-induced behavioral alteration (Moore, 2002; Thomas et al., 2005), symbiont-mediated alteration of host behavior might be more common among mutualistic associations than previously thought, particularly wherein symbiont transmission entails behavioral components.

Pest status of host stinkbugs is determined by their gut symbiotic bacteria

Closely related pest and nonpest plataspid stinkbugs

M. punctatissima (Figure 5.1A) is commonly found in mainland Japan, while *M. cribraria* (Figure 5.1B) is distributed across the southwestern islands of Japan. They are classified into different species on the basis of morphology. For example, *M. cribraria* is smaller in size and paler in color than *M. punctatissima*. However, they are no doubt very close genetically, considering that their mitochondria 16S rRNA genes showed over 99% sequence identity and that reciprocal crosses between the species produced viable F1 and F2 offspring (Hosokawa, unpublished data). The main host plants of *M. punctatissima* and *M. cribraria* are wild leguminous vines *P. lobata* and *P. montana*, respectively, while these insects occasionally utilize other leguminous plants. *M. punctatissima* has been known as a pest of soybean, pea, and other crop legumes. The insects often gregariously infest the plants, and without spraying, lay eggs and proliferate in the legume field. Meanwhile, *M. cribraria* scarcely causes such agricultural problems in Japan (Tomokuni, 1993).

What differs between pest and nonpest stinkbugs?

We evaluated the general performance of *M. punctatissima* and *M. cribraria* on potted soybean plants and pea pods. Both species grew normally to adult and laid fertilized eggs (Figures 5.16B–F). However, the egg hatch rates were strikingly different between the species: around 80% in *M. punctatissima* in contrast to only 50% in *M. cribraria* (Figure 5.16G). A characteristic mortality symptom was observed in the egg masses of *M. cribraria*, wherein many nymphs failed to escape from the eggshell and died (Figure 5.17B). These results indicated that the nonpest species *M. cribraria* suffers low egg hatch rate on the crop legumes, whereas the pest species *M. punctatissima* does not, which is probably relevant to their different pest status. Though utilized under laboratory conditions, the crop legumes are unsuitable host plants for *M. cribraria*.

Experimental symbiont exchange between pest and nonpest stinkbugs

In most of obligate endosymbiotic associations in insects, like those in aphids and tsetse flies, the host and the symbiont are structurally, functionally, and developmentally integrated into an almost inseparable biological entity (Douglas, 1998; Baumann et al., 2000; Shigenobu et al., 2000; Akman et al., 2002). Thus, it has been practically impossible to manipulate these obligate host–symbiont associations experimentally. However, the capsule-mediated transmission system in the plataspid stinkbugs enables such experiments despite the obligate nature of the symbiosis. The host eggs and the symbiont capsules

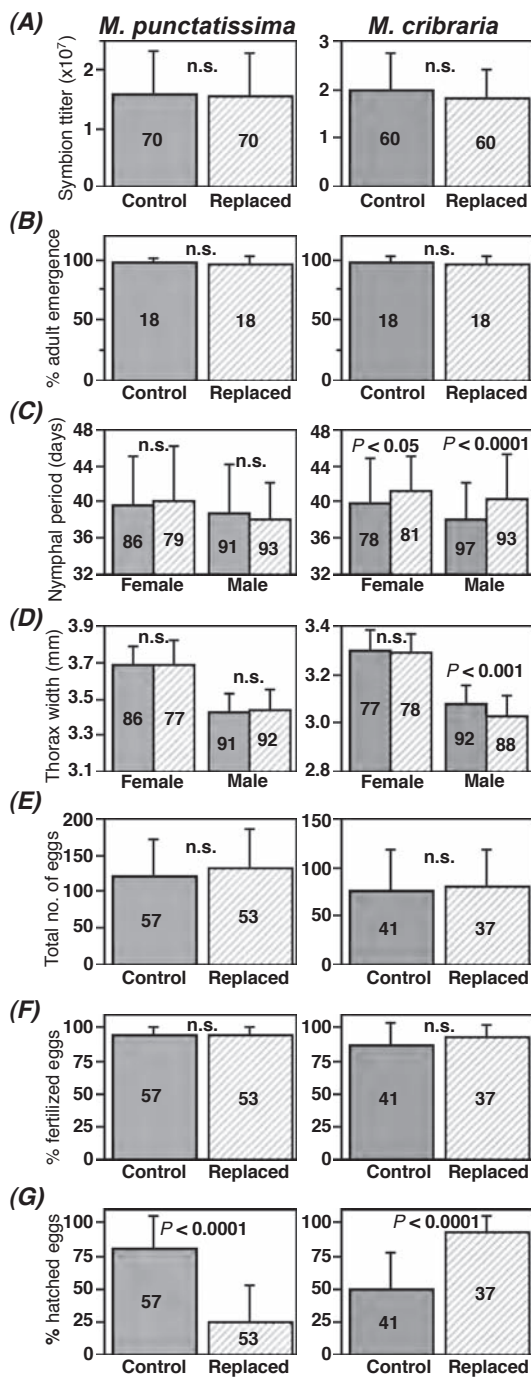


Figure 5.16 Fitness measurements of normal and symbiont-replaced plataspid stinkbugs. (A) Symbiont titer acquired by newborn nymphs, in terms of *groEL* gene copies. (B) Adult emergence rate (%). (C) Growth rate, in terms of nymphal period (days). (D) Adult body size, in terms of thorax width (mm). (E) Total number of eggs produced by an adult female. (F) Fertilization rate of eggs (%). (G) Hatch rate of eggs (%). (From Hosokawa, T., Kikuchi, Y., Shimada, M., and Fukatsu T. [2007]. *Proc. R. Soc. B.* **274**: 1979–1984. With permission.)

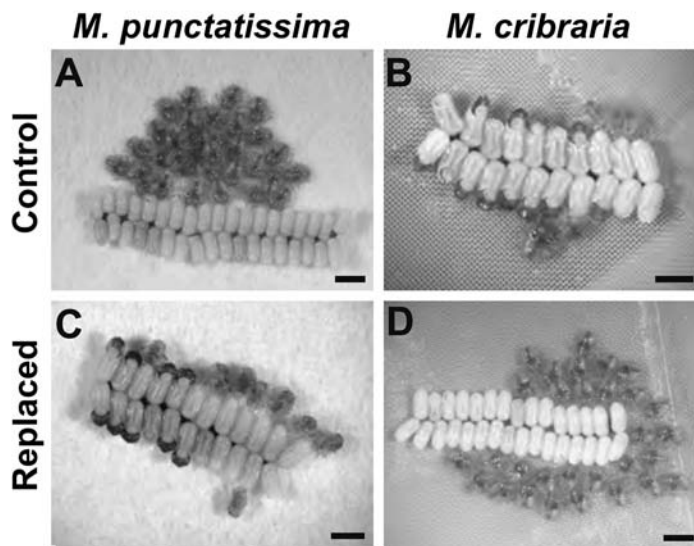


Figure 5.17 Mortality symptom observed with hatchlings of plataspid stinkbugs reared on the crop legumes. (A) and (C), *M. punctatissima*; (B) and (D), *M. cribraria*; (A) and (B), egg masses laid by normal females; (C) and (D), egg masses laid by symbiont-replaced females. Bars show 1 mm. (From Hosokawa, T., Kikuchi, Y., Shimada, M., and Fukatsu T. [2007]. *Proc. R. Soc. B.* **274**: 1979–1984. With permission.)

were separated by forceps under a binocular microscope, and the eggs of *M. punctatissima* were combined with the capsules of *M. cribraria*, and vice versa. The hatchlings probed the heterospecific capsules. Quantitative PCR assays confirmed that the nymphs certainly acquired the heterospecific symbiont cells, and the acquired amount was equivalent to that of the conspecific symbiont cells (Figure 5.16A).

Pest became nonpest and nonpest became pest after symbiont exchange

The symbiont-replaced insects grew normally to adults (Figure 5.16B-D). Although symbiont-eliminated adults of these stinkbugs suffered abnormal coloration and reduced body size (cf. Figures 5.10 and 5.11), the symbiont-replaced adults were almost indistinguishable from the control adults. In both the species, the eggs laid by the symbiont-replaced adults were certainly fertilized (Figure 5.16E), but egg hatch rates were strikingly different between the species: around 90% in *M. cribraria* in contrast to only 25% in *M. punctatissima* (Figure 5.16G). In the egg masses laid by the symbiont-replaced females of *M. punctatissima*, many nymphs failed to escape the eggshell and died (Figure 5.17C), which looked like the symptom found in the egg masses laid by the normal females of *M. cribraria* (Figure 5.17B).

Pest status of stinkbug determined by symbiont genotype

These results strongly suggested that the pest status of *M. punctatissima* is determined by the symbiont genotype rather than by the insect genotype. The mechanism whereby the symbiont from *M. punctatissima* can support the normal development of the host insects on

the crop legumes is currently unknown. Among the plataspid stinkbugs phylogenetically analyzed, *M. punctatissima* and *M. cribraria* are the most closely related (cf. Figure 5.9). The symbionts of *M. punctatissima* and *M. cribraria* are also very closely related: their 16S rRNA genes exhibited 99.9% (1308/1309 nucleotide sites) sequence identity, and their genome size was 0.8 Mb in common (cf. Figure 5.13). Plausibly, mutations occurring in the symbiont genome after the host speciation have modulated their capability of using different host plants, which predisposed the host insects to potentially become pest or not.

Insights into pest evolution, control, and management

Recently, it has been demonstrated that some facultative bacterial endosymbionts substantially affect various ecological traits of herbivorous insects including plant specialization (for details, see Chapter 7). The finding in the plataspid stinkbugs indicates that even obligate symbionts that play essential biological roles for their host may also influence plant specialization, and suggests that such symbionts could potentially be causal agents of emergent insect pests. It is currently unknown how prevalent similar cases of symbiont-mediated plant adaptation are in natural and agricultural ecosystems. In this context, it is of both evolutionary and practical importance to survey the correlation between symbiont genotypes and host races/biotypes/ecotypes in various insect-microbe symbiotic systems.

A number of agricultural, economical, and medical insect pests harbor symbiotic microorganisms (Bourtzis and Miller, 2003, 2006). In some of these cases, the symbionts have been suggested as possible agents for controlling the pests by using paratransgenic approach (Durvasula et al., 1997; Ben Beard et al., 2002), symbiont-derived population replacement (Dobson, 2003; Sinkins and Gould, 2006), and incompatible insect technique (Zabalou et al., 2004). Strikingly, it was reported that the most widely applied biological insecticide, *Bacillus thuringiensis*, is effective to lepidopteran larvae only when the insects harbor a gut microbial community (Broderick et al., 2006), indicating relevance of insect gut bacteria to pest control.

The gut symbiotic bacteria of the plataspid stinkbugs provide a model system for understanding the mechanisms underlying the symbiont-mediated pest evolution, which would potentially lead to novel means of pest control and management. Functional and genomic analyses of the stinkbug symbiont would lead to further insights into how the symbionts affect such ecological traits of the host insects.

Conclusion and perspective

By making full use of the unique symbiotic system of the plataspid stinkbugs, we have investigated as described above, and will investigate as follows, previously untouched aspects of the insect-bacterium mutualism. For example, egg/capsule ratios in an egg mass can be freely altered, by which the levels of maternal investment in symbiont transmission can be experimentally manipulated. By exchanging eggs and capsules between population, species, and genera of the insects, the extent of host-symbiont coevolution and coadaptation would be experimentally evaluated. Biochemical and nutritional analyses of isolated capsules would lead to understanding of the nature of symbiont inoculum upon vertical transmission. Isolated capsules can be the source of pure symbiont DNA needed for genome sequencing. We expect that, in addition to the aphid-*Buchnera*, tsetse-*Wigglesworthia*, and *Drosophila*-*Wolbachia* systems, the plataspid stinkbugs and their *Ishikawaella* symbionts provide an excellent model system for insect symbiosis studies.

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Endosymbiont that broadens food plant range of host insect

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Host plant specificity of herbivorous insects

Herbivorous insects are generally able to utilize a limited range of food plants. The host plant specificity is an important ecological trait for herbivores, not only determining their food and habitat but also strongly affecting their biological interactions with other organisms associated with the same plants. There are a number of topical research subjects related to the issue, such as adaptive mechanisms of insect biochemistry, physiology, and behavior; evaluating the adaptive potential of pest insects; evolution of ecological specialization, etc. (Futuyma and Peterson, 1985; Diehl and Bush, 1984). Even within the same insect species, remarkable variations in plant utilization have been frequently documented, wherein the ecologically and genetically distinct varieties are referred to as “biotype,” “host race,” or “ecotype,” offering model systems for studies on speciation (Gould, 1983; Futuyma and Peterson, 1985; Via, 2001).



Figure 6.1 Pea aphids, *Acyrtosiphon pisum*, on a seedling of broad bean, *Vicia faba*.

Pea aphid and host plant utilization

The pea aphid *Acyrtosiphon pisum* (Figure 6.1, Homoptera: Aphididae) feeds on several leguminous plants of the family Fabaceae, including peas, broad beans, alfalfa, clovers, vetches, and others. In the pea aphid, genetic variations in plant adaptation have been inferred by genetic structure analysis (Via, 1999, 2000; Simon et al., 2003; Frantz et al., 2005), performance analysis (Sandström and Pettersson, 1994; Via, 1999, 2000), behavioral analysis (Caillaud and Via, 2000; Del Campo et al., 2003; Ferrari et al., 2006), and QTL analysis (Hawthorne and Via, 2001). In these studies, plant adaptation of the pea aphid was generally attributed to the insect genotypes. However, a series of our studies revealed that a facultative endosymbiont strongly affects plant adaptation of the host aphid.

Endosymbiotic bacteria in pea aphid

Like most other aphid species, all pea aphid individuals possess an intracellular symbiotic bacterium, *Buchnera aphidicola*, in the cytoplasm of hypertrophied cells in the abdomen specialized for endosymbiosis, called mycetocytes or bacteriocytes (Buchner, 1965; Douglas, 1989, 1998). *Buchnera* provides the host aphid with essential amino acids and other nutrients that are scarce in the plant sap diet (Douglas, 1989, 1998, 2006), and deprivation of the symbiont by antibiotic or heat treatment results in retarded growth, sterility, and/or death of the host (Houk and Griffiths, 1980; Ohtaka and Ishikawa, 1991). Phylogenetically, the *Buchnera* symbionts of diverse aphids constitute a monophyletic group in the γ -subdivision of the *Proteobacteria* and cospeciate with their hosts (Unterman et al., 1989; Moran et al., 1993), indicating an ancient evolutionary origin of the endosymbiotic association. Because of the prevalence and importance, *Buchnera* is often referred to as the primary symbiont (P-symbionts) of aphids.

In addition to the P-symbiont *Buchnera*, a number of aphids harbor additional types of vertically transmitted endosymbiotic bacteria, which have been collectively referred to as secondary symbionts (S-symbionts) (Buchner, 1965; Fukatsu and Ishikawa, 1993, 1998; Fukatsu et al., 1998; Tsuchida et al., 2002). To date, five types of S-symbionts have been identified from the pea aphid: γ -proteobacterial *Serratia symbiotica* (also referred to as PASS or R-type), *Hamiltonella defensa* (PABS or T-type), and *Regiella insecticola* (PAUS or U-type) (Sandström et al., 2001; Darby et al., 2001; Russell et al., 2003; Haynes et al., 2003; Tsuchida et al., 2005; Moran et al., 2005); α -proteobacterial *Rickettsia* sp. (Chen et al., 1996; Sakurai et al., 2005); and *Spiroplasma* sp. (Fukatsu et al., 2001). Until recently, however, biological aspects of the S-symbionts in natural populations of the pea aphid have been poorly understood, which prompted us to extensively survey the endosymbiotic microbiota of the pea aphid in the field.

Endosymbiotic microbiota in natural populations of pea aphid

A total of 977 insects were collected from two major host plants of the pea aphid, the vetch *Vicia sativa* and the white clover *Trifolium repens*, at 96 localities covering the main islands of Japan. We conducted diagnostic PCR analysis targeting *Buchnera* and seven facultative symbiotic bacteria, *Serratia*, *Regiella*, *Hamiltonella*, *Rickettsia*, *Spiroplasma*, *Wolbachia*, and *Arsenophonus*. The extensive and systematic survey revealed that S-symbiont microbiota of Japanese pea aphids consisted of four bacterial components, *Serratia*, *Regiella*, *Rickettsia*, and *Spiroplasma*. Interestingly, the four S-symbionts exhibited their own characteristic geographic distribution patterns (Figure 6.2). In particular, *Regiella* exhibited a remarkable infection cline across mainland Japan, i.e., higher infection frequencies in northern Japan (Figure 6.2B).

Environmental factors relating to Regiella infection

It is of great interest how the characteristic distribution pattern of *Regiella* infection has been formed in Japanese populations of the pea aphid. Due to the long stretch of the Japanese archipelago, the climate remarkably varies across the country, resulting in different temperature, precipitation, and other environmental factors. These differences also affect the distribution of plants, i.e., vetch is absent in northern Japan because of cool climate, whereas white clover is found everywhere. Therefore, the possibility should be considered that *Regiella* distribution is relevant to these environmental factors.

First, we statistically analyzed the correlation between *Regiella* infection frequencies and those environmental data that were retrieved from meteorological stations nearby the collection sites of the aphid samples. The following three major environmental correlates were identified: host plant species, mean annual temperature, and mean annual precipitation (Table 6.1; Figure 6.3). Although speculative, several mechanisms are conceivable as to how these factors affect the infection frequency of *Regiella*. For instance, aphids infected with *Regiella* might perform better on white clover than on vetch. A cold climate might favor survival and reproduction of *Regiella*-infected insects. Under high humidity conditions where pathogenic microorganisms flourish, infection with *Regiella* might result in inferior survival of host insects. Based on the statistical approach, however, it is difficult to sort out which of these factors is actually responsible and to exclude involvement of other factors.

Next, we monitored temporal change of *Regiella* infection frequencies at three localities, Tsuchiura, Shimotsuma, and Yachiyo, where both vetch and clover were commonly found. At all three localities, *Regiella*-positive aphids were more frequently detected on

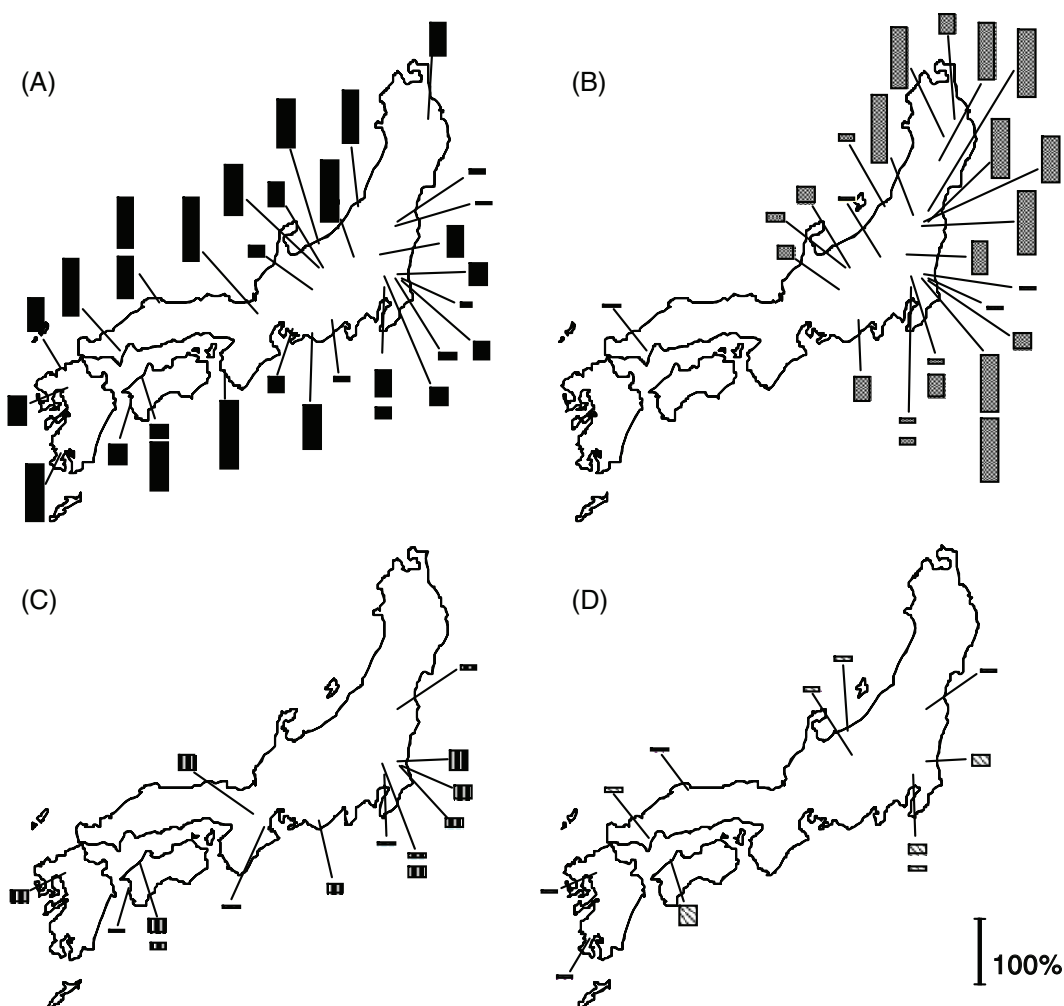


Figure 6.2 Geographic distribution and infection frequencies of secondary symbionts in Japanese populations of the pea aphid. (A) *Serratia*; (B) *Regiella*; (C) *Rickettsia*; (D) *Spiroplasma*. (From Tsuchida, T., Koga, R., Shibao, H., Matsumoto, T., and Fukatsu, T. [2002]. *Mol. Ecol.* **11**: 2123–2135. With permission.)

white clover than on vetch (Figure 6.4), which strongly suggested that the host plant is the principal environmental correlate of *Regiella* infection in the pea aphid populations. Of course, involvement of other factors, such as temperature and precipitation, cannot be ruled out. Although not significant, infection frequencies of *Regiella* on white clover slightly decreased toward the end of June at all three localities (Figure 6.4), which might be attributable to minor effects of these factors.

It should be noted that the frequent occurrence of *Regiella*-infected aphids on white and/or red clovers was not only observed in Japan but also in American and European populations of the pea aphid (Leonardo and Muir, 2003; Simon et al., 2003; Ferrari et al., 2004), corroborating the idea that the correlation is biologically meaningful.

Table 6.1 Analysis of Covariance (ANCOVA) for the Effect of Environmental Factors on *Regiella* Infection (Also See Figure 6.3)

Factor	<i>df</i>	<i>MS</i>	<i>F</i>	<i>p</i>
Mean annual temperature	1	0.783	7.89	0.0079
Mean annual precipitation	1	0.742	7.48	0.0095
Mean annual snowfall	—	—	—	—
Host plant species	1	0.879	8.86	0.0051
Mean annual temperature × Host plant species	—	—	—	—
Mean annual precipitation × Host plant species	1	0.474	4.78	0.0352
Mean annual snowfall × Host plant species	—	—	—	—
Error	37	0.100		
Model: $R^2 = 0.605057$, $F = 16.7031$, $df = 4, 37$, $p < 0.0001$				

Note: Dashes indicate exclusion of environmental variable from the model through stepwise regression procedure.

Source: From Tsuchida, T., Koga, R., Shibao, H., Matsumoto, T., and Fukatsu, T. (2002). *Mol. Ecol.* **11**: 2123–2135. With permission.

Why is Regiella infection predominant on clover?

For vertically transmitted microorganisms, increasing the fitness of infected individuals relative to uninfected individuals is an effective strategy for spreading and maintaining their infection in host populations (O'Neill et al., 1997; Fukatsu et al., 2000, 2001). In this context, the high frequency of *Regiella* infection on white clover might be accounted for by the mechanism that *Regiella* infection improves the fitness of the host aphid specifically on white clover. To test this hypothesis, it is essential to adopt experimental approaches in which environmental conditions and genetic backgrounds of the symbionts, aphids, and plants are strictly controlled in the laboratory.

How can effect of Regiella infection be evaluated in disymbiotic system?

Phenotypic effects of a symbiont infection should be evaluated by a comparison between infected and uninfected host insects under at least nearly identical genetic backgrounds. Such insect strains can be generated by three conventional techniques: (1) introgression (Bordenstein and Werren, 1998; Kondo et al., 2005), (2) transfection (Boyle et al., 1993; Chen and Purcell, 1997; Sasaki and Ishikawa, 2000; Fukatsu et al., 2001), and (3) curing (Wilkinson, 1998; Heddi et al., 1999). If the insect of interest is associated with a single symbiont species, these techniques generally work well. However, when two different symbionts coexist in the same insect as in the case of the pea aphid, it is difficult to selectively analyze the effects of one of the symbionts. For that purpose, elaborate experimental techniques are needed for adding or eliminating one of the symbionts selectively.

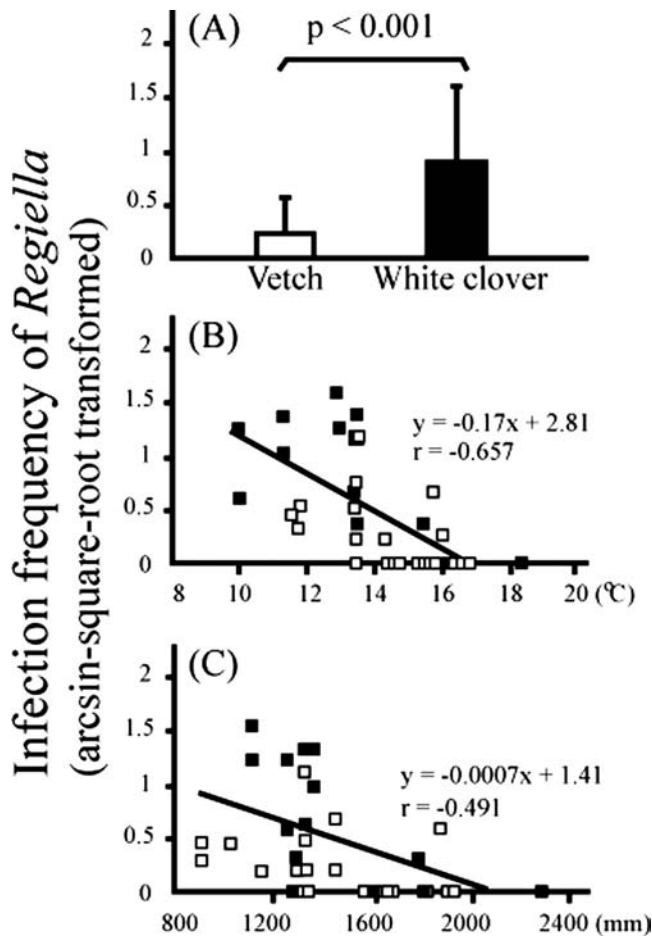


Figure 6.3 Environmental factors significantly correlated with the infection frequencies of *Regiella*. (A) Host plant species. (B) Mean annual temperature. (C) Mean annual precipitation. Also see Table 6.1. (From Tsuchida, T., Koga, R., Shibao, H., Matsumoto, T., and Fukatsu, T. [2002]. *Mol. Ecol.* **11**: 2123–2135. With permission.)

Morphology and localization of *Regiella*

Fluorescence *in situ* hybridization (FISH) analyses revealed that *Regiella* is found in three different locations in the aphid tissues: sheath cells, secondary mycetocytes, and hemolymph (Figure 6.5). In the hemolymph, *Regiella* cells were predominantly observed, whereas few *Buchnera* cells were detected (Figure 6.5B). Electron microscopy identified that the cell wall of *Buchnera* is reduced (Figure 6.6), which agreed with the previous electron microscopic observation (Hinde, 1971) and the genomic gene repertoire of *Buchnera* that lacks some genes for biosynthetic pathway of cell wall (Shigenobu et al., 2000). In contrast, the cell wall of *Regiella* was clearly observed (Figure 6.6) as in other facultative aphid endosymbionts *Serratia*, *Rickettsia*, and *Hamiltonella* (Fukatsu et al., 2000; Sakurai et al., 2005; Sandström et al., 2001; Moran et al., 2005).

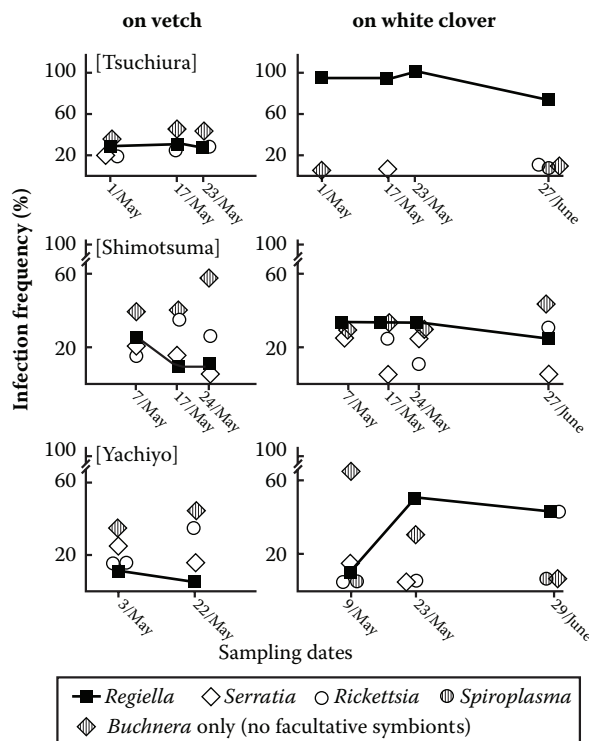


Figure 6.4 Infection frequencies of *Regiella* and other secondary symbionts on sympatric vetch and white clover, monitored from May to June 2002 at three localities, which are 30–50 km apart from each other, in central Japan. At each locality, 20 unwinged adult aphids per plant per date were analyzed. (From Tsuchida, T., Koga, R., and Fukatsu, T. [2004]. *Science* **303**: 1989. With permission.)

Techniques for enabling selective removal and selective transfer of *Regiella* infection

It turned out that *Regiella* has a well-developed cell wall, whereas *Buchnera* does not (Figure 6.6). Therefore, we expected that a drug that inhibits bacterial cell wall synthesis would selectively act on *Regiella* without affecting *Buchnera*. Based on this idea, we treated aphids of the *Regiella*-infected strain with an antibiotic, ampicillin, using a microinjection technique. As expected, we successfully obtained a *Regiella*-eliminated strain from the disymbiotic strain (Figure 6.7). The antibiotic-based technique for selective elimination of S-symbiont infections without affecting the essential endosymbiont was developed by us and has been used in many functional studies on aphid endosymbiosis (Koga et al., 2003, 2007; Leonardo, 2004; Sakurai et al., 2005; Leonardo and Mondor, 2006; Douglas et al., 2006; Simon et al., 2007).

The obligate symbiont *Buchnera* is exclusively endocellular, whereas *Regiella* occur not only endocellularly but also freely in the hemolymph (Figure 6.5). Thus, hemolymph injection can selectively transfer *Regiella* cells. Injection of hemolymph from infected insects into uninfected ones easily established a stable *Regiella* infection in the recipients, and the *Regiella* infection was vertically transmitted to the offspring of the recipient over 24 gen-

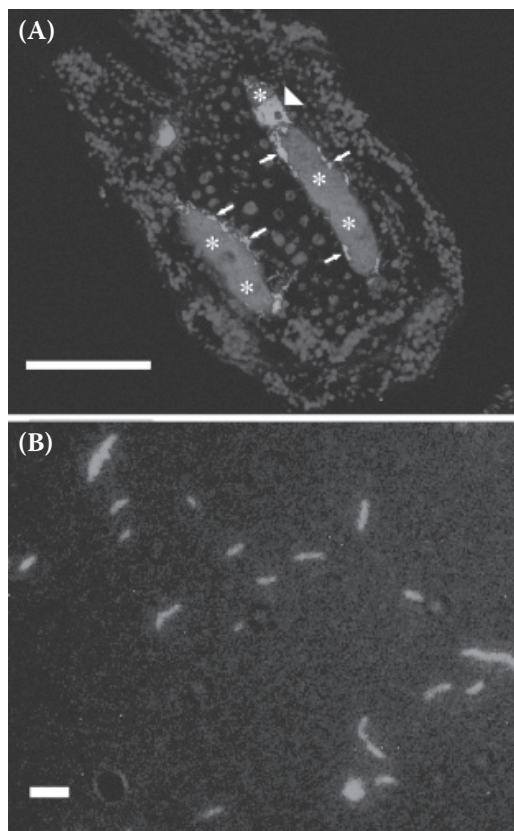


Figure 6.5 Fluorescence *in situ* hybridization of *Regiella* and *Buchnera* in an embryo of the *Regiella*-infected strain of the pea aphid. Specific probe for 16S rRNA of each symbiont was used. (A) A tissue section of a pea aphid embryo. *Regiella* in a secondary mycetocyte (arrowhead) and sheath cells (arrows), which were in close association with primary mycetocytes harboring *Buchnera* (asterisks). Bar, 50 μ m. (B) A hemolymph preparation from adult pea aphids. *Regiella* cells were predominantly detected. Bar, 10 μ m. (From Tsuchida, T., Koga, R., Meng, X.Y., Matsumoto, T., Fukatsu, T. [2005]. *Microb. Ecol.* **49**: 126–133. With permission.)

erations (Table 6.2). The experimental techniques for selective S-symbiont elimination and transfer enabled us to strictly evaluate the fitness effects of *Regiella* infection.

Endosymbiotic impact of Regiella on plant adaptation of pea aphid

We generated aphid strains that are genetically identical and differ only in *Regiella* infection. Using the selective elimination technique, we obtained a *Regiella*-free strain TU^{tamp}, and a *Regiella*-infected control strain TU^{tdw} that had been injected with distilled water instead of the antibiotic. Although these strains exhibited similar levels of fecundity on vetch, the *Regiella*-eliminated strain lost almost 50% of fecundity compared with the infected strain on white clover (Figure 6.8, top). We reintroduced *Regiella* into the TU^{tamp} strain by injecting hemolymph from the infected strain TU^t, to generate an infected strain TU^{tamp/TU^t}. A control uninfected strain TU^{tamp/TU^{tamp}} was prepared by injecting hemolymph from TU^{tamp}

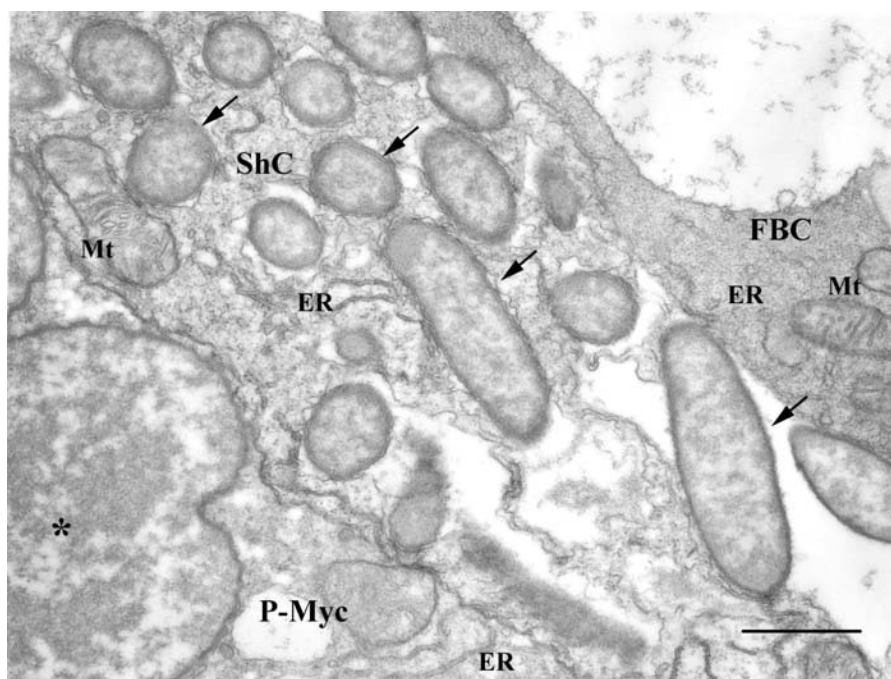


Figure 6.6 Electron microscopy of pea aphid embryos of the *Regiella*-infected strain. Rod-shaped *Regiella* cells are seen in the cytoplasm of a sheath cell, located between a fat body cell (upper right) and a primary mycetocyte harboring *Buchnera* (lower left). Arrows and asterisks indicate *Regiella* and *Buchnera* cells, respectively. Bars, 2 μ m. Abbreviations: ER, endoplasmic reticulum; FBC, fat body cell; Mt, mitochondrion; P-Myc, primary mycetocyte; ShC, sheath cell. (From Tsuchida, T., Koga, R., Meng, X.Y., Matsumoto, T., Fukatsu, T. [2005]. *Microb. Ecol.* **49**: 126–133. With permission.)

into the same strain TU^{tamp}. The reintroduction of *Regiella* resulted in an almost complete recovery of fecundity in the recipient aphids on white clover (Figure 6.8, bottom). These results unequivocally indicated that *Regiella* infection improves the fitness of the host aphid specifically on white clover.

The positive fitness effects of *Regiella* infection on the host aphid were much greater on white clover than on vetch (Figure 6.8), indicating that *Regiella* may benefit the host aphid preferentially on white clover. In the field, vetch is more densely and frequently colonized by pea aphids than white clover (personal observation). The pea aphid clones with only *Buchnera* generally exhibit significantly better performance on vetch than on white clover (Figure 6.9). These results suggest that white clover is not an optimal host plant for pea aphid without the secondary symbiont. It appears plausible that *Regiella*-infected aphids better utilize the otherwise unsuitable host plant, white clover, resulting in higher infection frequency of *Regiella* on white clover as observed in Japanese natural populations (Figures 6.3 and 6.4).

Possible mechanisms of symbiont-mediated plant adaptation

At present, the mechanisms whereby *Regiella* improves performance of host aphid specifically on white clover are totally unknown. One possible mechanism is that *Regiella* is

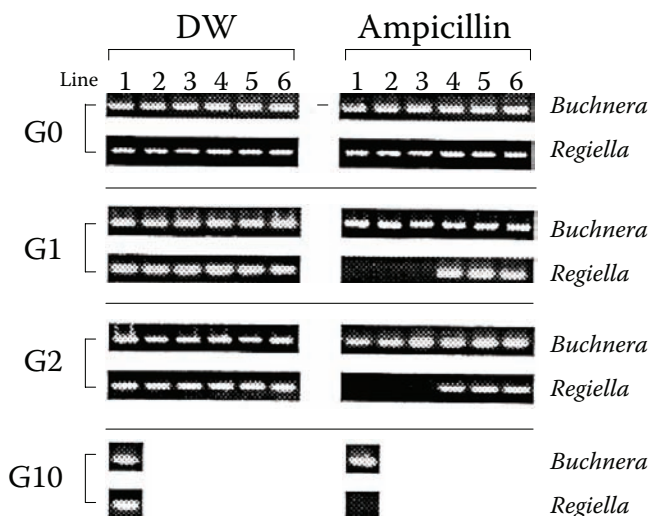


Figure 6.7 Diagnostic PCR for confirming selective elimination of *Regiella* infection from pea aphid. Lanes 1–6, control aphid lines injected with distilled water; lanes 7–12, treated aphid lines injected with ampicillin at a dose of 1 µg/mg body weight. In a part of the treated lines (3/33 lines), *Regiella* infection disappeared in the offspring, whereas *Buchnera* infection was not affected. Complete elimination of *Regiella* infection was confirmed over 10 successive generations using diagnostic PCR. G0, treated generation; G1, offspring of G0; and so forth.

Table 6.2 Artificial Transfer of *Regiella* from Strain TUt to Strain AIST by Injection of Hemolymph

Injected Lines	Injected Generation						Successive Generations		
	5–6 (8–9) ^a	7–8 (10–11)	9–10 (12–13)	11–12 (14–15)	13–14 (16–17)	15– (18–)	1 ^c	2 ^d	24 ^e
AIST-TUt	0/4 ^b	0/6	2/6	4/6	6/6	8/8	10/10	10/10	20/20
AIST-TUt 2	0/4	0/6	3/6	4/6	6/6	8/8	10/10	10/10	20/20
AIST-TUt 3	0/4	0/6	2/6	5/6	6/6	8/8	10/10	10/10	20/20

^a Days after injection (days after birth).

^b Number of infected offspring/number of offspring examined.

^c Established from a nymph deposited 12–13 days after injection of the injected generation.

^d Established from a newborn nymph of generation 1.

^e About 6 months after injection.

Source: From Tsuchida, T., Koga, R., Meng, X.Y., Matsumoto, T., and Fukatsu, T. (2005). *Microb. Ecol.* **49**: 126–133. With permission.

involved in the physiological adaptation of the host aphid to the chemical composition of plant phloem sap. It has been shown that amino acid composition in phloem sap differs among plant species (Ziegler, 1975; Weibull, 1990; Douglas, 1993; Sandström and Pettersson, 1994; Wilkinson and Douglas, 2003). Experiments with artificial diet rearing systems have shown that dietary amino acid composition certainly affects the aphid performance (Srivastava and Auclair, 1983; Prosser and Douglas, 1992; Sandström, 1994; Karley et al.,

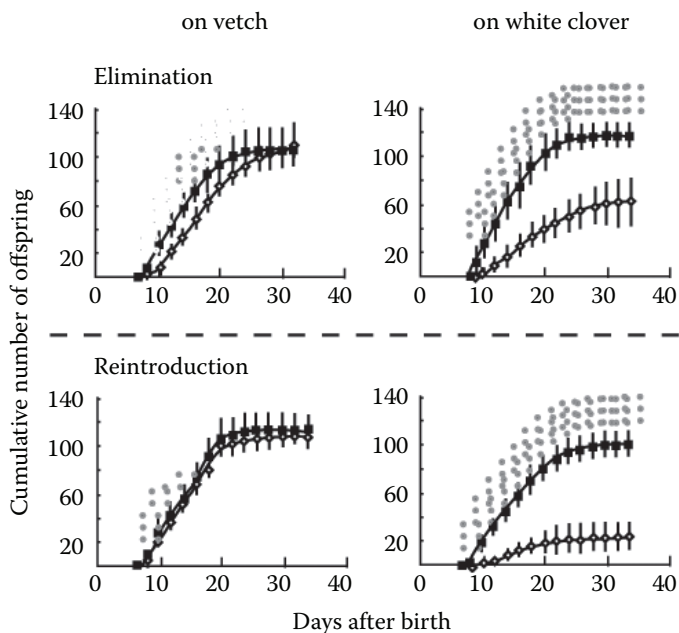


Figure 6.8 Performance of *Regiella*-infected and uninfected aphids on vetch and white clover. Top panels: naturally *Regiella*-infected strain $TU_{t^{dw}}$ (filled squares), and *Regiella*-eliminated strain $TU_{t^{amp}}$ (open diamonds). Bottom panels: *Regiella*-eliminated strain $TU_{t^{amp}}/TU_{t^{amp}}$ (open diamonds), and *Regiella*-reintroduced strain $TU_{t^{amp}}/TU_{t^{dw}}$ (filled squares). Dots and bars indicate means and SD. Asterisks indicate statistically significant differences (Mann-Whitney U test: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). (From Tsuchida, T., Koga, R., and Fukatsu, T. [2004]. *Science* **303**: 1989. With permission.)

2002). Moreover, it has been suggested that dietary amino acid composition and concentration affect density and phenotypic effects of endosymbionts (Wilkinson et al., 2001, 2007; Chandler et al., 2008). Hence, it appears plausible that the amino acid quality and quantity in the phloem sap are relevant to the influence of *Regiella* on aphid performance on different plant species. Amino acid compositions in the phloem sap of white clover and vetch are unknown, but it is conceivable that *Regiella* might provide essential amino acids that *Buchnera* cannot sufficiently synthesize from the phloem sap of white clover. However, Douglas et al. (2006) conducted experiments using a range of artificial diets, in which amino acid compositions varied, and found no evidence that *Regiella* provides essential amino acids.

Alternatively, plant allelochemicals might be possible determinants of aphid performance on the different plants. White clover contains an array of bioactive secondary metabolites including cyanogenic glycosides (Williams, 1987), which were suggested to be effective against herbivores including aphids (Dritschilo et al., 1979). *Regiella* might be able to detoxify the plant allelochemicals, as *Symbiotaphrina kochii* does in the cigarette beetle (Dowd, 1991).

To address which of these and other hypotheses is justifiable, further experimental studies on the physiological contribution of *Regiella* to the host aphid are needed. It will be pivotal to analyze the phloem sap composition of white clover and vetch and investigate

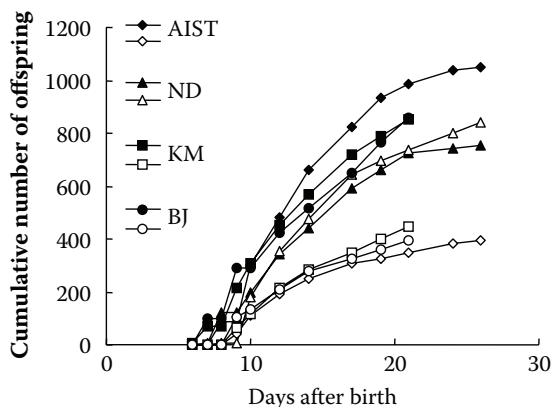


Figure 6.9 Cumulative number of offspring of four pea aphid clones on vetch (filled symbols) and white clover (open symbols). All these aphid strains harbor *Buchnera* only. The aphids were reared on each of the plants in groups of ten, respectively. The experiments were repeated twice on each plant for each clone, and an average of two experiments is indicated. Experimental condition: 16L8D, 20°C.

the nutritional physiology of *Regiella*-infected and *Regiella*-free aphids using chemically defined artificial diets.

Variation in effects of Regiella infection on plant adaptation

Leonardo (2004) conducted a similar study using two clones of the pea aphid and found that, in contrast to our results, removal of *Regiella* did not affect aphid performance on white clover. There are several possibilities that can account for these apparently conflicting results. First, we should consider the genetic differences between the Japanese and American aphid strains used in these studies. The strains that Leonardo used showed increased mortality on alfalfa and increased fecundity on clover (Leonardo and Muir, 2003; Leonardo, 2004). The alfalfa-specific mortality was also observed with *Serratia*-infected aphids, which belong to one of the genotypes harboring *Regiella* (Leonardo, 2004). These results may suggest that those aphid clones adapt to clover due to their nuclear genome. In contrast, the Japanese strain we used exhibit no alfalfa-related mortality (unpublished data), suggesting that the Japanese clone is genetically distinct from the American clone. A recent study conducted by Ferrari et al. (2007) highlighted the importance of aphid nuclear backgrounds in combination with *Regiella* infection. They conducted experiments using five aphid clones, which were created by artificial injection of *Regiella*. One clone showed increased performance on red clover by *Regiella* infection, whereas four clones exhibited no effect on performance.

Second, the conflict might be due to the genetic variation of *Regiella* strains used in these experiments. Even when identified as belonging to the same species, different bacterial strains are sometimes vastly different genetically and phenotypically to each other. For instance, *Escherichia coli* K12 is nonpathogenic, whereas *E. coli* O157:H7 is virulently pathogenic, and their genome constitutions are remarkably different (Perna et al., 2001). Such variations of the facultative symbiont might account for the different performance of Japanese and American pea aphids on white clover.

Third, the differences in plant sources used in these studies might be relevant. White clover consists of many cultivars and ecotypes, whose chemical compositions are different. For instance, white clover cultivars exhibit remarkable genetic variations in production of the cyanogenic glycosides linamarin and lotaustralin, and the activating enzyme linamarase, which hydrolyses the cyanogenic glucosides to liberate toxic hydrocyanic acid (Williams, 1987). It should be noted, of course, that these possibilities are not mutually exclusive.

Possible horizontal transfer of Regiella

It has been suggested that facultative symbionts of insects, including *Regiella*, have extended their host range by occasional horizontal transfers. Plant phloem sap, parasitoid wasps, predators, and interspecific copulation and insemination have been suggested as potential horizontal transmission routes for aphid endosymbionts (Sandström et al., 2001; Darby et al., 2001; Russell et al., 2003; Darby and Douglas, 2003; Tsuchida et al., 2005; Moran and Dunbar, 2006). In addition to broadening of the host plant range enabled by *Regiella*, S-symbionts play important ecological roles for their host insects, such as tolerance to high temperature conferred by *Serratia* and *Hamiltonella* (Montllor et al., 2002; Russell and Moran, 2006); resistance to parasitoid wasps caused by *Hamiltonella* and *Serratia* (Oliver et al., 2003, 2005, 2008); resistance to parasitic fungi conferred by *Regiella* (Scarborough et al., 2005); and induction of winged and sexual morphs modified by *Regiella* (Leonardo and Mondor, 2006). Hence, if such “evolutionary innovator” S-symbionts are horizontally transferred across different lineages and species, it would have great impact on the evolution of the host insects.

Several experimental studies have failed to detect intraspecific horizontal transfers of S-symbionts in aphids via the host plant (Chen and Purcell 1997; Chen et al., 2000; Darby and Douglas, 2003). However, Darby and Douglas (2003) suggested that an S-symbiont, *Hamiltonella*, could be horizontally transferred via artificial diet. Moreover, considering that S-symbiont infection can easily be transferred by hemolymph injection (Table 6.2; Chen and Purcell, 1997; Chen et al., 2000; Fukatsu et al., 2001; Koga et al., 2003; Oliver et al., 2003, 2005, 2006; Scarborough et al., 2005; Tsuchida et al., 2004, 2005; Russell and Moran, 2006), horizontal transfer of S-symbionts, at least within the same species, might be vectored by oviposition of parasitoid wasps.

Moran and Dunbar (2006) experimentally demonstrated that, during sexual reproduction, *Regiella* and *Hamiltonella* could be transmitted to progeny not only maternally but also paternally. Considering that a male can mate with many females and sometimes attempt mating with heterospecific females (Moran and Dunbar, 2006), interspecies symbiont transfer might occur even when fertilization is not possible. In this context, sexual morph production modified by *Regiella* infection (Leonardo and Mondor, 2006) might be relevant to increase the probability of interspecific horizontal transfer of the symbiont.

Several studies have suggested that horizontal transfers of S-symbionts can potentially occur across different aphid species. Chen and Purcell (1997) experimentally transferred *Serratia* and *Rickettsia* from pea aphid *Acyrtosiphon pisum* to a related aphid *A. kondoi* by injection of infected hemolymph. Darby and Douglas (2003) suggested that *Hamiltonella* can be transferred from *A. pisum* into *Aphis fabae* via diet. Russell and Moran (2005) artificially transferred S-symbionts from three aphid species, *Myzocallis* sp. (infected with *Arse-nophonus*), *Macrosiphum euphorbiae* (with *Regiella*), and *Aphis craccivora* (with *Hamiltonella*), into *A. pisum*, using hemolymph transfer. In these studies, the establishment of new infection was prevented by low transmission efficiency and negative fitness effects on the hosts. Tsuchida et al. (2006) examined the possibility of horizontal transfer between three aphid

species, *A. pisum*, *Aphis craccivora*, and *Megoura crassicauda*, living on the same host plant, *Vicia faba*. The results suggested that the occurrence of interspecific horizontal transfers of the S-symbionts must be rare between these sympatric aphid species. However, an artificially transferred *Regiella* strain from *A. pisum* was stably maintained in a novel host *M. crassicauda* with 100% fidelity for over 50 generations. Hence, some S-symbionts are potentially able to expand their host range beyond species border and be stably maintained in the novel host.

Other cases of symbiont-mediated host plant adaptation to be found

It has been suggested that symbiotic microorganisms are potentially involved in a part of genetic variations in plant utilization of herbivores (Diehl and Bush, 1984; Jones, 1984; Douglas, 1989). To date, however, only a few studies have empirically demonstrated the roles of endosymbionts to plant adaptation of insects. Eisenbach and Mittler (1987) identified a maternally inherited trait in an aphid, *Schizaphis graminum*, that overcomes the cultivar's resistance, although it was not shown whether the factor is an endosymbiont or not. We found that plant adaptation of the pea aphid is governed by an S-symbiont, *Regiella* (Tsuchida et al., 2004). Recently, Hosokawa et al. (2007) demonstrated that pest status of a stinkbug is principally determined by its gut bacterial symbiont (also see Chapter 5). These findings suggest that such symbionts could be causal factors of novel pest emergence.

Thus far, to our knowledge, no other studies have reported such symbiont-mediated insect adaptation to specific food plants. However, considering that insect–plant relationships are generally influenced by diverse microorganisms ranging from pathogens to mutualists (Barbosa et al., 1991; Vega and Blackwell, 2005; Colvin et al., 2006), there is no reason to believe that symbiont-mediated plant adaptation is restricted to a small group of insects. It is of great importance not only for basic biology of plant–insect interactions but also for pest herbivore management to investigate various insect–microbe symbiotic systems.

Perspectives

Our studies started from the survey of endosymbiotic microbiota in natural populations of the pea aphid, and have led to the discovery of the unexpected phenomenon: endosymbiont-mediated broadening of food plant range. Now we are working on a number of research subjects, as follows:

1. Elucidation of the physiological mechanisms of *Regiella*-mediated plant adaptation: To understand the molecular and chemical basis of the phenomenon, we are analyzing chemical components of the plant phloem sap, and comparing honeydew of the *Regiella*-infected and uninfected aphids feeding on the plants. Once the candidate compounds are identified, their involvement will be tested by using chemically defined artificial diet rearing systems. The metabolic pathway will be determined by tracer experiments, and expression and activity of enzymes responsible for the metabolism of the compounds will be analyzed. Furthermore, constructing *Buchnera*-free aphid by using selective elimination method (Koga et al., 2003, 2007) would provide novel insights into the issue. On the basis of these analyses on fitness effects and metabolic changes, the molecular and chemical interactions between *Regiella*, *Buchnera*, and host aphid involved in the plant adaptation would be unveiled.
2. Genome analysis of *Regiella*: For understanding the evolutionary process and molecular mechanism of the symbiont-mediated plant adaptation, we are now analyzing

the whole genome of the *Regiella* strain whose involvement in plant adaptation has been experimentally demonstrated.

3. Analyses of insect genotype, symbiont type, and evolutionary history of the association: To understand genetic basis of host plant specialization in the pea aphid and influence of the symbiont, we are now analyzing genetic structure of the pea aphid and the symbiont infection status using high-resolution genetic markers. By comparing genetic relatedness within and between host races of the pea aphid from Europe, America, and Asia, we will be able to gain insights into the evolutionary history of host specialization and evolutionary origin of the symbiont infection. The advance of the pea aphid genome project will further accelerate our genetic approaches to the symbiotic association between the aphid and the diverse endosymbiotic bacteria.
4. Interactions between host genotypes and symbiont genotypes that affect life history parameters of aphids: Influence of *Regiella* on several life history parameters including plant adaptation will be examined under different combinations of aphid genotypes of and symbiont genotypes. Such analyses would reveal aphid–symbiont interactions, and functional sharing between aphid genotype and symbiont genotype in ecological contexts.
5. Spatiotemporal infection dynamics of *Buchnera* and *Regiella* in the same host aphids on different host plants: The density and localization of the obligate and facultative symbionts will be examined on vetch and white clover throughout the aphid development. Such analyses would provide an important clue to understanding the multilevel interactions between *Regiella*, *Buchnera*, aphid, and host plants involved in the symbiont-mediated plant adaptation.
6. Biological effects of *Regiella* infection on novel host: After artificial transfer into novel host aphids, we will examine the localization and population of *Regiella* and *Buchnera*, and also effects on their ecological traits, especially plant adaptation. Such experiments would provide insights into the impact of the horizontal transfer of the symbiont on the evolution of aphids in general.

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Insect–bacterium mutualism without vertical transmission

Yoshitomo Kikuchi and Takema Fukatsu

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Introduction

A number of insects establish endosymbiotic associations with microorganisms, which have had great impact on the host insect's evolution. Some symbiotic microbes improve the host's metabolism, which enables the host to utilize nutritionally insufficient food materials and to explore novel ecological niches. Other symbiotic microbes, like members of the genus *Wolbachia*, manipulate the host's reproduction in a selfish manner, causing drastic phenotypes such as cytoplasmic incompatibility (CI) and parthenogenesis, which might lead to reproductive isolation among host populations and ultimately to host speciation. In mutualistic associations like aphid–*Buchnera* and tsetse–*Wigglesworthia* endosymbioses, the interacting partners generally exhibit strict interdependence with each other: symbiont-deficient host insects suffer nymphal mortality and/or sterility (Bourtzis and Miller, 2003; Buchner, 1965), whereas symbiotic microbes cannot survive outside the host insects. In such obligate associations, therefore, failure in symbiont transmission/acquisition directly leads to death of both of the symbiotic partners.

To ensure the infection of such benevolent symbionts to the next generation, insects have evolved a variety of elaborate mechanisms for symbiont transmission. Intracellular symbionts of obligate nature harbored in mycetocytes, like *Buchnera* in aphids, are generally passed to the next generation by transovarial transmission, where the symbiotic

bacteria are directly delivered into oocytes in the process of oogenesis (Buchner, 1965; Miura et al., 2003). Many intracellular symbionts of rather parasitic nature, such as *Wolbachia* and other facultative symbionts in various insects, are also transmitted in a trans-ovarial manner (Bourtzis and Miller, 2003; Frydman et al., 2006). Meanwhile, in insects associated with extracellular gut symbionts, posthatch mechanisms for symbiont transmission such as “egg smearing” and “coprophagy” are commonly found (Buchner, 1965). In egg smearing known from, for example, longicorn beetles and anobiid beetles, eggs are superficially contaminated with symbiotic microbes during oviposition, and hatchlings acquire the symbionts by consuming or probing the eggshell (Breitsprecher, 1928; Jones et al., 1999; Schomann, 1937). In coprophagy known from termites and their relatives, juveniles feed on anal excrements of adult individuals and acquire symbionts (Honigberg, 1970; Inoue et al., 2000). In addition, milk-gland transmission in tsetse flies (Aksoy et al., 1997) and capsule transmission in plataspid stinkbugs (see Chapter 5) represent unique mechanisms of symbiont transmission. In summary, a variety of mechanisms for symbiont transmission have evolved among diverse insect taxa, all of which reported thus far are vertical modes.

Recently, a novel mechanism for symbiont transmission was discovered in some stinkbugs of the family Alydidae (Hemiptera: Heteroptera). In this symbiotic system, a mutualistic symbiont is present in the soil of the insect habitat, and the stinkbugs acquire the symbiont not by vertical transmission but from surrounding environment every generation. The mechanism, so-called environmental transmission, has been well-known from plant-microbe endosymbioses such as legume-*Rhizobium* and alder-*Frankia* nitrogen-fixing relationships, but not known from insect endosymbioses previously. In this chapter, we first present an introduction to the bacterial associations in heteropteran stinkbugs, next review our current knowledge on the biology of the unique alydid symbiosis, and finally discuss the evolutionary aspects of the microbial symbiosis without vertical transmission in insects, pointing out future directions.

Overview of bacterial symbiosis in stinkbugs

Stinkbug taxonomy and its bacterial symbiosis

Insects belonging to the suborder Heteroptera are commonly called “stinkbugs,” because they have a habit to exude foul-smelling fluid when disturbed. To date, over 38,000 species have been described in the Heteroptera (Schuh and Slater, 1995), which is rated among the largest insect groups with incomplete metamorphosis. Their habitats range from grassland to aquatic environment, and their feeding habits are also diverse. Some species feed on vertebrate blood, whereas others prey on other arthropods, suck mycelia of fungi, or feed on plants. Taxonomically, the heteropteran bugs are grouped into seven infraorders: the Enicocephalomorpha, the Dipsocoromorpha, the Gerromorpha, the Nepomorpha, the Leptopodomorpha, the Cimicomorpha, and the Pentatomomorpha (Schuh and Slater, 1995). Phylogenetic analyses on the basis of molecular and morphological data have suggested that the Heteroptera is a well-defined monophyletic group in the Hemiptera (Ouvrard et al., 2000; Schuh, 1979; von Dohlen and Moran, 1995; Wheeler et al., 1993), wherein each of the seven infraorders form a monophyletic group, respectively (Schuh, 1979; Wheeler et al., 1993).

Of the seven infraorders, symbiotic bacteria have been reported from the Cimicomorpha and the Pentatomomorpha (Buchner, 1965; Dasch et al., 1984; Glasgow, 1914). In the Cimicomorpha, blood-sucking species representing the families Reduviidae (assassin

bugs) and Cimicidae (bedbugs) harbor endosymbiotic bacteria in gut cavity, inside gut epithelial cells, or in specialized mycetocytes (Buchner, 1965; Dasch et al., 1984; Glasgow, 1914). In the Pentatomomorpha, almost all members, except for the predatory Asopinae and the mycophagous Aladoidae, are phytophagous, most of which possess symbiotic bacteria in their alimentary tract. In these plant-sucking species, conspicuous sacs or outgrowths, called caeca or crypts, are usually developed at a posterior region of the midgut (so-called midgut 4th section), whose lumen is populated by a number of specific bacterial cells (Buchner, 1965; Dasch et al., 1984; Glasgow, 1914). In most cases except the family Acanthosomatidae, the crypts are open into the midgut main-tract (Buchner, 1965; Goodchild, 1963). In some of the stinkbug species, it has been demonstrated that experimental elimination of the symbiont causes retarded growth and nymphal mortality of the host insects (Abe et al., 1995; Buchner, 1965; Chang, 1974; Fukatsu and Hosokawa, 2002; Hill et al., 1976; Hosokawa et al., 2006; Huber-Schneider, 1957; Müller, 1956; Schorr, 1957), suggesting important biological roles of the symbiotic bacteria for the hosts.

Recently, symbiotic bacteria of several stinkbug species have been microbiologically characterized using molecular phylogenetic approaches. *Rhodococcus rhodnii*, belonging to the bacterial phylum *Actinobacteria*, has been identified as gut symbiont of the hematophagous assassin bug *Rhodnius prolixus* (Beard et al., 2002). Gamma-proteobacterial symbionts have been characterized from the families Cimicidae, Pentatomidae, and Plataspidae (Fukatsu and Hosokawa, 2002; Hosokawa et al., 2006; Hysa and Aksoy, 1997; Prado et al., 2006), but these symbionts do not form a monophyletic group. In addition, facultative or parasitic endosymbionts of the genus *Wolbachia* have been commonly found in diverse heteropteran stinkbugs (Kikuchi and Fukatsu, 2003).

Mechanisms of symbiont transmission

As mentioned above, three major types of symbiont transmission (transovarial transmission, egg smearing, and coprophagy) have been known from diverse insects. In the Heteroptera, all three mechanisms are adopted by different groups: transovarial transmission in the family Cimicidae associated with mycetocyte symbionts (Buchner, 1965); egg smearing in the families Pentatomidae, Acanthosomatidae, and others (Abe et al., 1995; Prado et al., 2006; Rosenkranz, 1939); coprophagy in *Triatoma* assassin bugs (the family Reduviidae) and in the families Cydnidae and Coreidae (Huber-Schneider, 1957; Schorr, 1957). In addition, a unique and elaborate transmission mechanism called “capsule transmission” has been reported from the family Plataspidae, in which symbiont-filled particles called “symbiont capsules” are deposited with eggs, and hatchlings probe the content of the capsules to acquire the symbiont (Fukatsu and Hosokawa, 2002; Hosokawa et al., 2005; Hosokawa et al., 2006; Müller, 1956; Schneider, 1940; see also Chapter 5).

In the capsule-mediated symbiosis of the Plataspidae, the symbiont phylogeny was perfectly concordant with the host insect phylogeny (Hosokawa et al., 2006; see also Chapter 5), suggesting that the symbiotic association was established in the common ancestor of the stinkbugs and has been stably maintained solely by vertical transmission over evolutionary time. Such a host–symbiont coevolutionary pattern was also observed in the family Acanthosomatidae, wherein the symbiotic bacteria are vertically transmitted by egg smearing (Kikuchi et al. 2007, unpublished data).



Figure 7.1 A female adult of the bean bug *Riptortus pedestris*. (Photo courtesy of S. Moriya.)

Characterization of Burkholderia symbiont in alydid stinkbugs

Members of the stinkbug family Alydidae, called broad-headed bugs, are distributed worldwide and include a number of pest species for leguminous and gramineous crops (Schaefer and Panizzi, 2000). In Japan, the alydid stinkbugs *Riptortus pedestris* (Figure 7.1), formerly known as *R. clavatus* and recently renamed by Kikuhara (2005), and *Leptocorisa chinensis* are notorious pests of soybean and rice, respectively (Schaefer and Panizzi, 2000; Tomokuni et al., 1993). The bean bug and the rice bug can be easily reared in the laboratory on soybean seeds and rice grains, respectively. In the stinkbug species, we found well-developed crypts at a posterior region of the midgut (Figure 7.2) (Kikuchi et al., 2005). Light and electron microscopy revealed that the lumen of the crypts is densely populated by rod-shaped bacteria (Figure 7.3).

Cloning and sequencing of 16S rRNA gene amplified from the midgut crypts revealed that a single bacterial species dominated in the midgut microflora. BLAST searches and molecular phylogenetic analyses demonstrated that the gut bacteria are closely related to each other (with slight sequence diversity), belonging to the genus *Burkholderia* in the β -*Proteobacteria* (Figure 7.4). Fluorescent *in situ* hybridization confirmed that the *Burkholderia* symbiont specifically localized in the lumen of the midgut crypts (Figure 7.2F). Prevalence of the *Burkholderia* infection was consistently high, 95%–100%, in natural populations of the alydid stinkbugs. All these results indicated that *R. pedestris* and *L. chinensis* harbor β -proteobacterial symbiont of the genus *Burkholderia* in the lumen of the midgut crypts (Kikuchi et al., 2005).

The high prevalence of the *Burkholderia* symbiont in the host populations suggests an intimate association of the symbiotic bacteria with the host stinkbugs. In obligate mutualistic associations like aphid–*Buchnera* and tsetse–*Wigglesworthia* systems, the symbionts have been maintained by strict vertical transmission over evolutionary time, resulting in

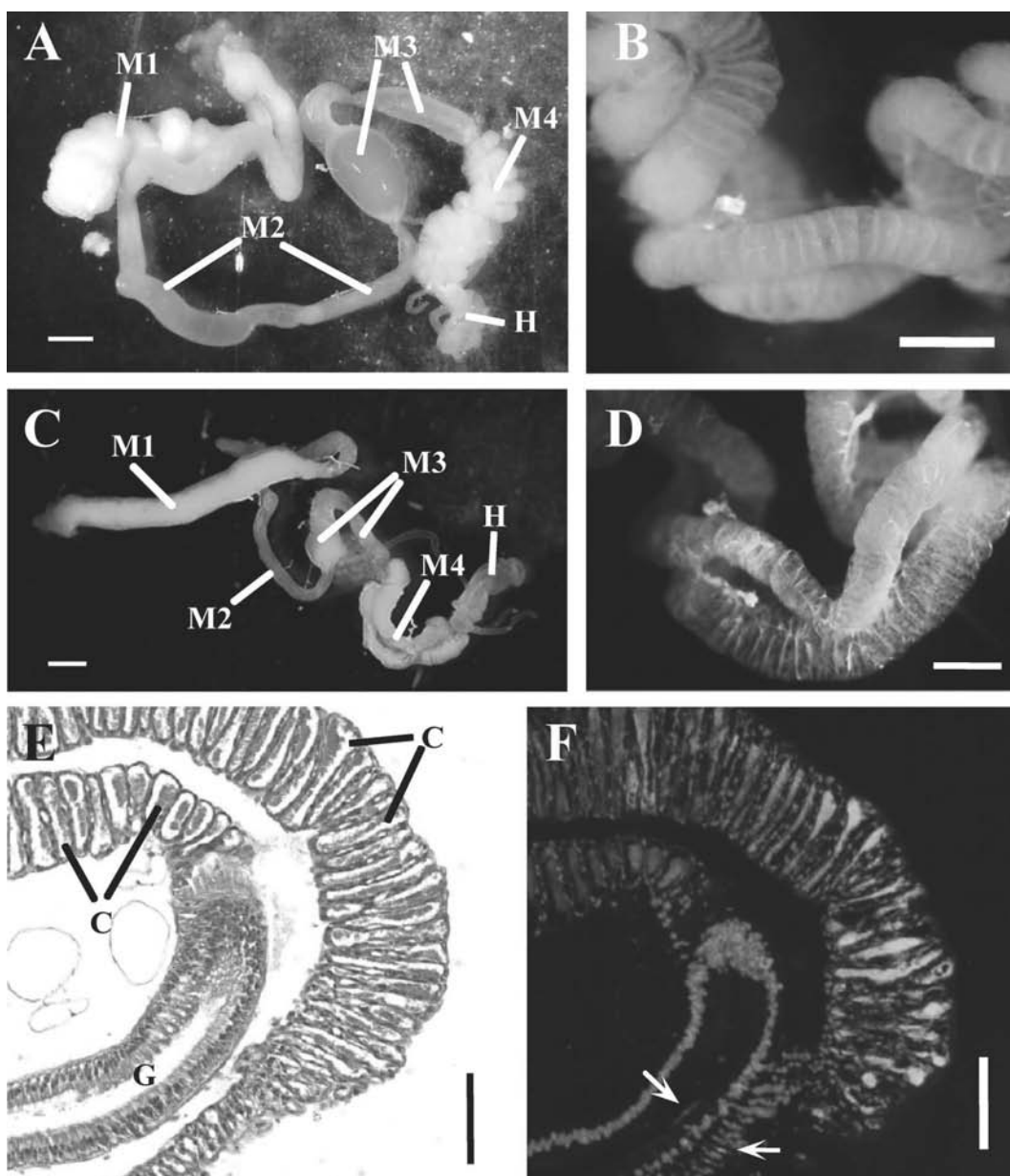


Figure 7.2 (Color figure follows p. 238.) Midgut organization of *R. pedestris* and *L. chinensis*. A dissected midgut of (A) *R. pedestris* and (C) *L. chinensis*. Enlarged image of midgut fourth section with crypts of (B) *R. pedestris* and (D) *L. chinensis*. (E) A tissue section of midgut crypts of *R. pedestris*, stained with hematoxylin and eosin. (F) Fluorescent *in situ* hybridization of a tissue section of midgut crypts of *R. pedestris*, targeting 16S rRNA of the *Burkholderia* symbiont. Green signals are due to the *Burkholderia*-specific probe Cy3-Alsymb16S. Blue signals are nuclei of the host cells visualized by DAPI. Arrows indicate the *Burkholderia* signals in the main tract of the midgut and those in the ducts connecting the crypts with the main tract. Bars, 0.5 mm in (A) and (B), 0.2 mm in (C) and (D), and 100 μ m in (E) and (F). Abbreviations: C, crypt; G, gut; M1, midgut first section; M2, midgut second section; M3, midgut third section; M4, midgut fourth section (symbiotic organ); H, hindgut. (Modified from Kikuchi, Y., Meng, X.Y., and Fukatsu, T. [2005]. *Appl. Environ. Microbiol.* 71: 4035–4043. With permission.)

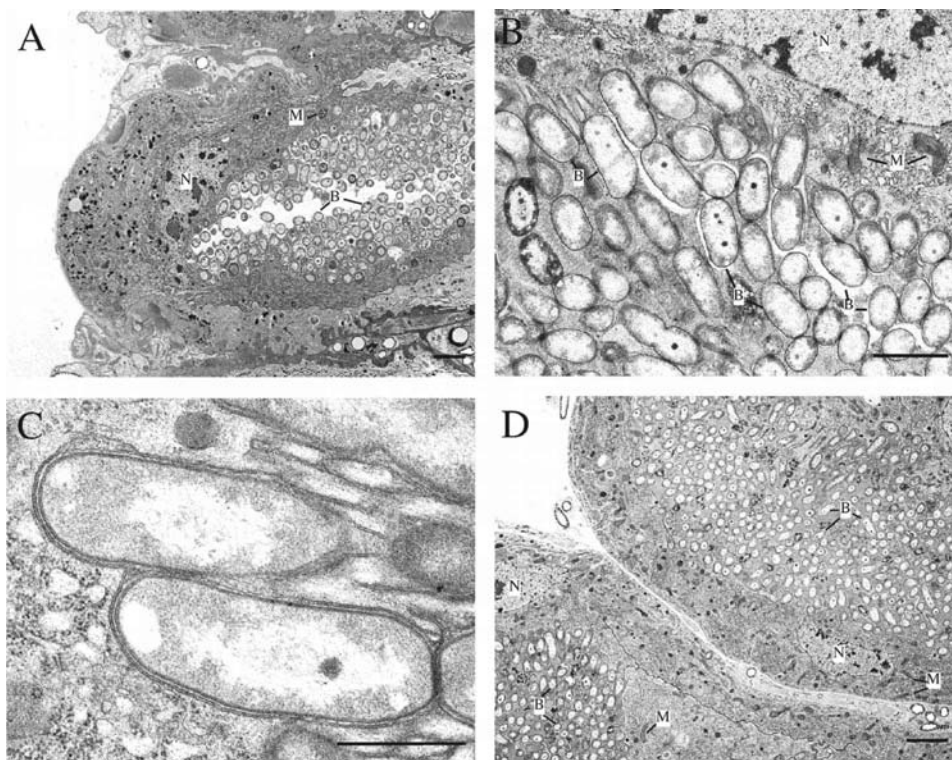


Figure 7.3 Transmission electron microscopy of the midgut crypts of *R. pedestris* and *L. chinensis*. (A–C) *R. pedestris*; (D) *L. chinensis*. (A) A crypt of *R. pedestris* harboring many rod-shaped bacteria. (B) An enlarged image of the interface between the lumen and the epithelium of the crypt. The bacteria are present only in the lumen. (C) An enlarged image of the rod-shaped bacteria. Well-developed cell wall and a particle-like structure are seen. (D) A crypt of *L. chinensis* similarly harboring many rod-shaped bacteria. Bars, 2 μm in (A) and (D), 1 μm in (B), and 300 nm in (C). Abbreviations: B, symbiotic bacterium; M, mitochondrion; N, nucleus. (From Kikuchi, Y., Meng, X.Y., and Fukatsu, T. [2005]. *Appl. Environ. Microbiol.* **71**: 4035–4043. With permission.)

host–symbiont phylogenetic congruence (Chen et al., 1999; Clark et al., 2000). In the Heteroptera, such host–symbiont coevolutionary patterns have been reported from the Plataspidae (Hosokawa et al., 2006) and the Acanthosomatidae (Kikuchi et al., 2007, unpublished data). The symbionts identified from natural populations of *R. pedestris* and *L. chinensis* certainly constituted a well-defined monophyletic group in the genus *Burkholderia*, but the phylogenetic relationship of the symbiont was far from the pattern of cocoladogenesis or cospeciation, neither reflecting the host systematics nor the geographic distribution of the host insects (Figure 7.4) (Kikuchi et al., 2005). The intermingled phylogenetic pattern suggested the possibility that horizontal transmission of the symbiont might have occurred frequently between populations and species of the alydid stinkbugs. Occasional coinfections with two strains of the *Burkholderia* symbiont (cf. *R. pedestris* OK and *L. chinensis* KM; see Figure 7.4) are also suggestive of occurrences of such horizontal transmission. If the extracellular symbiont is vertically transmitted by a posthatch transmission mechanism

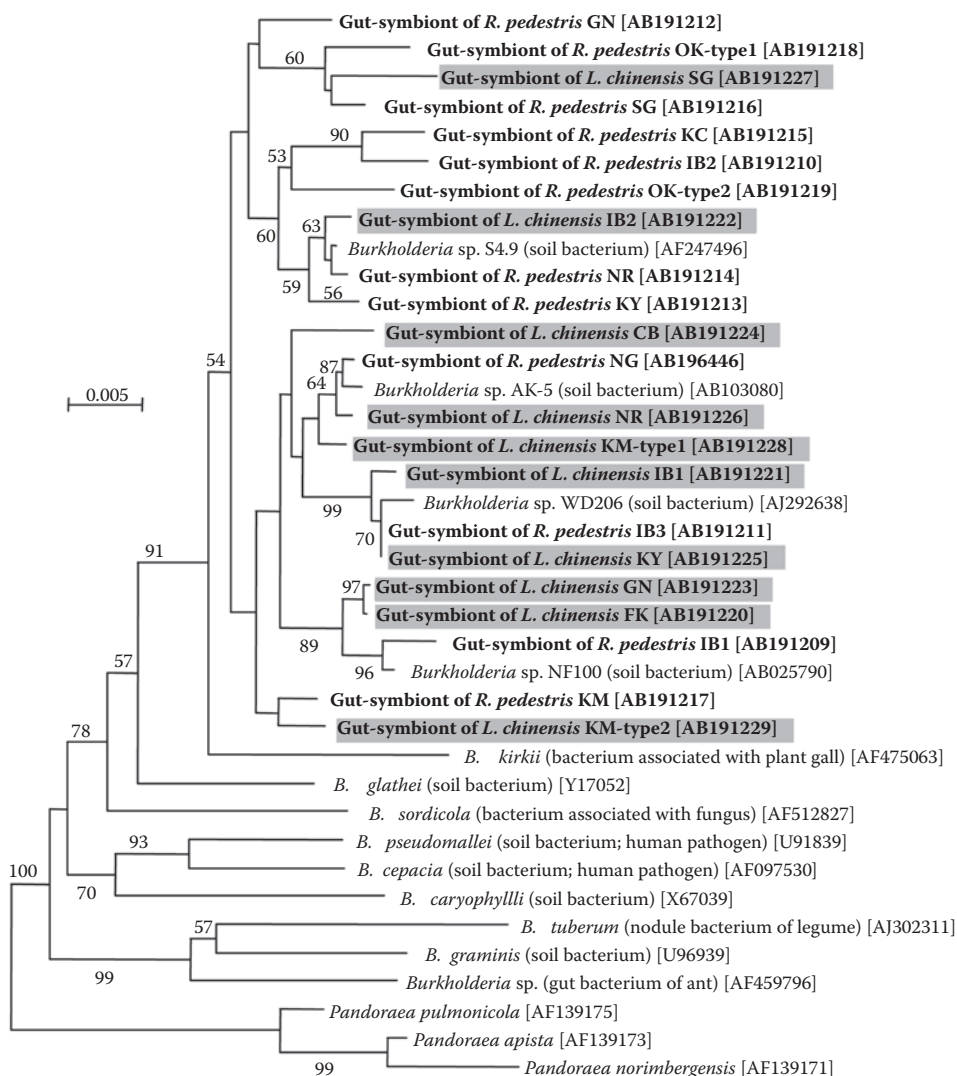


Figure 7.4 Phylogenetic relationship of the *Burkholderia* symbionts from *R. pedestris* and *L. chinensis* on the basis of 16S rDNA sequences. A neighbor-joining tree of 1,403 unambiguously aligned nucleotide sites is shown. The sequences from *R. pedestris* and *L. chinensis* (shaded) are represented in bold. The stinkbug samples were collected from CB, Chiba; FK, Fukushima; GN, Gunma; IB1, Ibaraki (Mito); IB2 Ibaraki (Tomobe); IB3, Ibaraki (Tsukuba); KC, Kochi; KM, Kumamoto; KY, Kyoto; NG, Nigata; NR, Nara; OK, Okinawa; SG, Saga, Japan. *Pandoraea* spp. were used as outgroup taxa for the genus *Burkholderia*. The bootstrap values higher than 50% are depicted at the nodes. In parentheses are shown the biological features of the *Burkholderia* strains. Nucleotide sequence accession numbers are shown in brackets. (Modified from Kikuchi, Y., Meng, X.Y., and Fukatsu, T. [2005]. *Appl. Environ. Microbiol.* 71: 4035–4043. With permission.)

Table 7.1 Detection Rates of the *Burkholderia* Symbiont in *R. pedestris* Broods

Pair No.	% Symbiont Detection (Positive/Total)			<i>p</i> ^b
	(a) Egg	(b) Rearing with Parents in Clean Case ^a	(c) Rearing without Parents on Soybean Pot ^a	
Pair #1	0 (0/31)	0 (0/22)	100 (20/20)	<0.0001
Pair #2	0 (0/23)	0 (0/25)	100 (18/18)	<0.0001
Pair #3	0 (0/30)	0 (0/21)	96 (24/25)	<0.0001
Pair #4	0 (0/30)	0 (0/19)	82 (19/23)	<0.0001
Pair #5	0 (0/30)	0 (0/20)	82 (23/28)	<0.0001

^aNewly emerged adult insects were subjected to diagnostic PCR detection of the symbiont.

^bStatistical significance of the difference between (b) and (c) analyzed by Fisher's exact probability test.

like egg smearing and coprophagy, symbiont exchange between conspecific and hetero-specific individuals could accidentally occur under certain conditions.

Meanwhile, the promiscuous patterns of the symbiont phylogeny can be accounted for by an alternative mechanism. Considering that soil-derived *Burkholderia* isolates such as strains S4.9, AK-5, WD206, and NF100 (Friedrich et al., 2000; Hayatsu et al., 2000; Nogales et al., 2001; Takenaka et al., 2003) are placed in the same clade of the *Burkholderia* symbionts of the alydid stinkbugs (Figure 7.4), the possibility emerges that the alydid symbionts might have a free-living phase outside the host body in the soil environment.

Transmission mechanism of Burkholderia symbiont: not vertical transmission but environmental acquisition

We first examined the bean bug *R. pedestris* for the possibility of vertical transmission. When 144 eggs were harvested from field-collected, infected parents and were subjected to diagnostic PCR with specific primers for the *Burkholderia* symbiont, all the eggs were identified as symbiont-negative (Table 7.1). When 107 newborn nymphs were reared in clean plastic cages together with their infected parents, the hatchlings neither probed the egg surface nor sucked the excrement of their parents, and none of them got infected with the *Burkholderia* symbiont until adulthood (Table 7.1). These results suggested that neither egg smearing nor coprophagy occurs in the alydid stinkbug, leading to an unexpected idea that the alydid stinkbug does not transmit the symbiont vertically (Kikuchi et al., 2007). It should be noted that the *Burkholderia* symbiont was frequently (38/40 = positive/total observed) detected in the rhizosphere of soybean plants, and, surprisingly, almost all juveniles reared on potted soybean plants acquired the *Burkholderia* symbiont even in the absence of their infected parents (Table 7.1). These results strongly suggested that the stinkbug nymphs acquire the symbiont from surrounding environment every generation.

To confirm the possibility of environmental acquisition, rearing experiments by using sterilized soybean plants and a cultured strain of the *Burkholderia* symbiont were performed. It should be noted that, as expected from occurrence of the symbiont in soil environment, we successfully established a culture strain of the *Burkholderia* symbiont from the midgut crypts of the insect (Figure 7.5) (Kikuchi et al., 2007). Sterilized soybean bottles, in which soybean plants were grown aseptically, were inoculated with the cultured symbi-

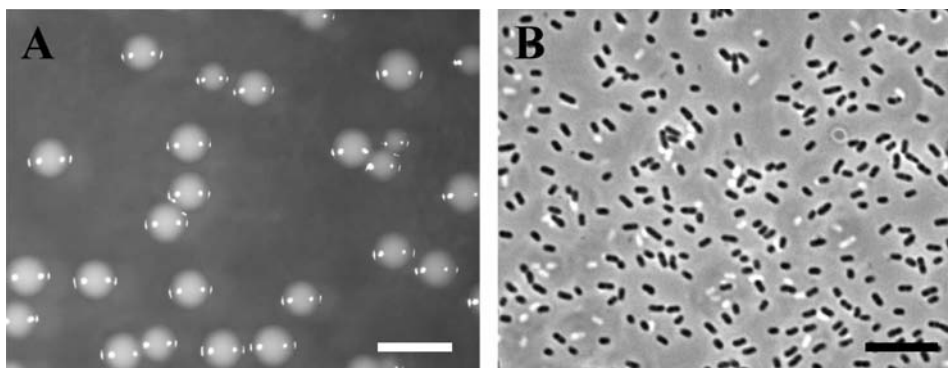


Figure 7.5 *Burkholderia* symbiont isolated from midgut crypts of *R. pedestris*. (A) Colonies of the *Burkholderia* symbiont on LB (Luria-Bertani) medium. (B) Microscopic image of cultured *Burkholderia* symbiont cells. Bars, 2 mm in (A), and 10 μ m in (B).

ont, and uninfected hatchlings of *R. pedestris* were reared in the bottles until adulthood. In the symbiont-inoculated bottles, all insects acquired the *Burkholderia* symbiont (100%, positive/total observed = 93/93), while no infection established in the control bottles without symbiont inoculation (0%, 0/83), indicating that the stinkbug nymphs are certainly able to acquire the symbiont environmentally.

At the beginning, the promiscuous host–symbiont relationship in the alydid stinkbugs was quite puzzling, but now we understand that the evolutionary pattern is a natural outcome of the environmental symbiont acquisition.

Beneficial nature of Burkholderia symbiont

To be stably maintained in host insect populations, endosymbiotic bacteria have developed several evolutionary strategies. Obligate symbionts like *Buchnera* in aphids and *Wigglesworthia* in tsetse flies adopt the “mutualist” strategy, where the symbionts are maintained in the host populations by undertaking pivotal biological roles for the host metabolism and improving the host fitness (Bourtzis and Miller, 2003; Douglas, 1998). Facultative symbionts like *Wolbachia*, *Spiroplasma*, *Cardinium*, and *Rickettsia* adopt the “reproductive manipulator” strategy, where the symbiont infections can spread in the host populations by means of reproductive manipulation, such as CI, parthenogenesis, feminization, and male killing (Bourtzis and Miller, 2003; O’Neill et al., 1997). Almost all of these well-studied insect endosymbionts are vertically transmitted through the host generations.

Then how does the *Burkholderia* symbiont attain the high, nearly 100%, prevalence in the alydid populations despite the absence of vertical transmission? When fitness parameters were compared between symbiont-infected and uninfected individuals of *R. pedestris*, all body size parameters (body length, thorax width, and abdomen width) examined were significantly larger in the former than in the latter (Figure 7.6) (Kikuchi et al., 2007), indicating that the *Burkholderia* symbiont plays beneficial biological roles for the host insect. However, the symbiont infection is not essential for the host survival and reproduction. At least under laboratory conditions, we were able to maintain aposymbiotic insects for several generations (Kikuchi et al., 2007). On the other hand, the symbiotic bacteria exhibited

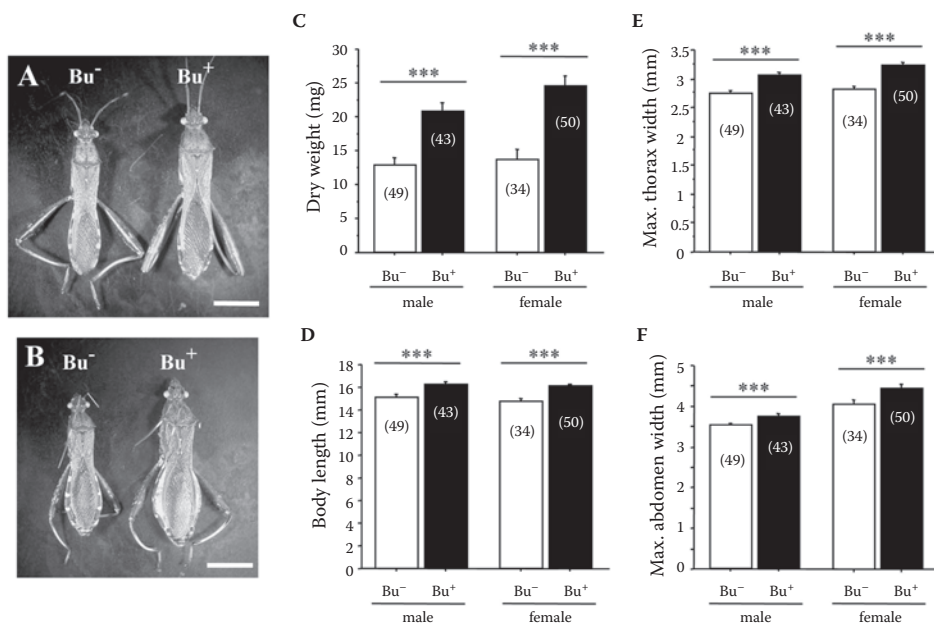


Figure 7.6 Comparison of fitness parameters between the *Burkholderia*-infected (Bu⁺) and uninfected (Bu⁻) adult insects of *Riptortus pedestris*. Photo images: (A) male; (B) female. Bars, 0.4 mm. (C) Dry weight; (D) body length; (E) maximum thorax width; (F) maximum abdomen width. Asterisks indicate statistically significant differences (Analysis of deviance: ***, $p < 0.0001$). (From Kikuchi, Y., Hosokawa, T., and Fukatsu, T. [2007]. *Appl. Environ. Microbiol.* **73**: 4308–4316. With permission.)

high levels of infection fidelity in the insect juveniles under several experimental conditions: 91% (positive/total observed = 104/114) in the soybean-pot rearing experiments and 100% (93/93) in the soybean-bottle rearing experiments (Kikuchi et al., 2007). Therefore, the high prevalence of the *Burkholderia* symbiont in natural populations of the alydid stink-bugs is probably ensured by the synergy between the selective, efficient infection and the positive effects on the host fitness.

Alydid-Burkholderia symbiosis as an insect analogue of plant-microbe symbioses

In summary, the bean bug *R. pedestris* acquires the beneficial symbiont not vertically but environmentally from the soil every generation. The alydid-*Burkholderia* association is quite different from typical insect-microbe endosymbioses in that the association entails no vertical transmission.

Almost all animals, including insects, harbor diverse microbial communities in their alimentary tract. Most of the gut microbes are probably commensalistic and/or rather parasitic, but some members of the microflora may affect the host organisms beneficially (Dethlefsen et al., 2007; Dillon and Dillon, 2004; Ley et al., 2006). These gut microbes are acquired either postnatally from the surrounding environment or vertically from the parents (Dillon and Dillon, 2004; Ley et al., 2006). In this context, it is conceivable that, even in more sophisticated gut symbiotic associations, beneficial bacteria could be acquired

from the environment. Actually, in the pheromonal symbiosis of desert locusts (Dillon and Charnley, 2002) and in the nutritional symbiosis of flower thrips (de Vries et al., 2001), environmentally acquired gut bacteria were reported to play substantial biological roles. In the case of *R. pedestris*, a specific soil bacterial clade is selectively incorporated into a specialized gut structure, establishes a stable and exclusive infection, attains nearly 100% prevalence in natural host populations, and significantly contributes to the host fitness. We consider this alydid–*Burkholderia* symbiosis as the first unequivocal case in which an insect acquires a specific bacterial symbiont of beneficial nature from the environment.

Apart from the insect endosymbiotic systems, however, specific associations of this type are broadly found in nature. In the marine ecosystem, for example, the squid–*Vibrio* bioluminescent symbiosis (Nyholm and McFall-Ngai, 2004), the coral–dinoflagellate photosynthetic symbiosis (Muscatine, 1973; Trench, 1993), the tubeworm–chemoautotroph nutritional symbiosis (Nussbaumer et al., 2006; Peek et al., 1998), and many others entail environmental symbiont acquisition during developmental course every generation. In the terrestrial ecosystem where insects are dominant, environmental acquisition of mutualistic symbiont has been known from the plant–microbe symbioses such as legume–*Rhizobium* and alder–*Frankia* nitrogen-fixing relationships (Benson and Silvester, 1993; Denarie et al., 1992) and mycorrhizal symbiosis in many higher plants (Bolan, 1991; Simard and Durall, 2004). In such plant–microbe symbioses, the symbionts are able to live freely in the soil environment, exclusively infect and colonize specialized symbiotic organs like nodules and mycorrhiza, and improve the host fitness. In this context, the stinkbug–*Burkholderia* relationship could be regarded as an insect analogue of the plant symbioses with the soil-associated microbes.

What benefits does Burkholderia symbiont receive from symbiosis?

It is of interest why the symbiotic bacteria maintain the symbiotic association with the bean bug in spite of their capability of free-living. The midgut crypt is an exclusive, physiologically stable niche for the *Burkholderia* symbiont, which no doubt constitutes a beneficial aspect of the association for the symbiont side. In the squid–*Vibrio* symbiosis, the host squids periodically excrete the symbionts from the light organ to refresh the bioluminescent activity, resulting in dominance of the symbiotic *Vibrio* in the surrounding oceanic environment (Lee and Ruby, 1994; Nyholm and McFall-Ngai, 2004). In the case of the *Rhizobium* symbiosis, the symbiotic bacteria are released from root nodules to the environment after death of the hosts (reviewed in Denison, 2000), which provides the symbiont source for infection to the next host generation. In the symbiotic systems without vertical transmission, the association-release cycle may be involved in the enhancement of the symbiont fitness. In this context, the FISH image of the stinkbug crypts, showing the symbiont signals in the midgut main-tract (Figure 7.2F), appears meaningful: this may suggest the possibility that the symbiont is occasionally excreted from the crypts into the environment with host feces.

Evolution of stinkbug–Burkholderia symbiosis

The evolutionary origin of intracellular symbiotic bacteria harbored in mycetocytes of various insects has been suggested as gut bacteria that colonized in the alimentary tract of the ancestral insects and played beneficial roles for them (Buchner, 1965). A number of studies have certainly shown that many mycetocyte-associated, obligate insect symbionts are phylogenetically related to enteric bacteria (reviewed in Baumann, 2005; Moran and Wer-

negreen, 2000). Although speculative, the evolutionary process has been presumably as follows: among diverse bacteria in the gut microflora, a competitive and beneficial bacterial species became dominant in the gut environment; structures for retaining the beneficial bacterium, such as cryptic organs, developed in the host alimentary tract, facilitating the host-symbiont association; mechanisms for vertical transmission of the bacterium evolved, further enhancing their interdependency; the bacterium was incorporated and harbored in host cells, further stabilizing their association and facilitating metabolic, physiological, and developmental interactions between the partners. In this context, the *Burkholderia* symbiont of the bean bug *R. pedestris*, which exclusively colonizes in the midgut crypts and is environmentally acquired every host generation, may represent a primitive stage of the evolutionary course from gut bacterium to highly specialized mycetocyte symbiont.

Theoretically, biological relationships between host organisms and their microbial partners, including pathogens, parasites, and mutualists, have been thought to depend on their transmission modes: horizontal transmission across different host lineages tends to facilitate the virulence of the associates, whereas vertical transmission through host generations tends to attenuate the virulence, potentially leading to the evolution of mutualism (Axelrod and Hamilton, 1981; Dieckmann et al., 2002; Ewald, 1987; Fine, 1975; Herre et al., 1999; Sachs et al., 2004; Yamamura, 1993). A number of theoretical studies have shown that vertical transmission is pivotal for evolution of mutualisms, at least under straightforward assumptions (Law and Dieckmann, 1998; Lipsitch et al., 1996; Yamamura, 1993; Yamamura, 1996), and many experimental studies have verified that pathogens diminish their virulence under the conditions of vertical infection (Bergstrom et al., 1999; Bull et al., 1991; Stewart et al., 2005). These theoretical frameworks generally agree with the evolutionary patterns observed in typical insect-microbe mutualisms, such as aphid-*Buchnera* symbiosis, wherein the association is maintained by vertical transmission. However, a number of mutualisms, including legume-*Rhizobium* symbiosis, squid-*Vibrio* symbiosis, and this alydid-*Burkholderia* symbiosis, look, at least superficially, to contradict with the theoretical expectations.

Several theoretical studies (Genkai-Kato and Yamamura, 1999; Wilkinson and Sherratt, 2001) have revealed the following conditions and factors that can promote the evolution of mutualism without vertical transmission: (1) vertical transmission of the symbiont incurs some cost for the host; (2) exploitation by the symbiont negatively affects the host; (3) the host controls the vertical transmission process; and (4) the host utilizes waste products of the symbiont. It is of interest to examine whether each of these conditions applies to the stinkbug-*Burkholderia* association. The condition (1) leads to a testable hypothesis that the symbiont infection at egg or early nymphal stages may be detrimental to the host stinkbug. The condition (2) could be verified by measuring the relationship between the infection densities of the symbiont and the fitness effects on the host. Concordant with the condition (3), the host stinkbug is likely to govern the vertical transmission mechanism, on the grounds that the midgut crypts develop even in the absence of the symbiont (Kikuchi et al., 2007). The condition (4) seems to apply to many insect-microbe mutualisms, in which the host utilizes symbiont-produced nutrients that are essential for the host but not necessary for the symbiont (Baumann et al., 2000; Douglas, 1998).

Conventionally, mutualistic associations without vertical transmission has been thought to be evolutionarily unstable, on the grounds that the symbiotic system is susceptible to invasion by microbes of different genotypes. It has been pointed out that symbiont heterogeneity within an individual host tends to exacerbate resource competitions among different symbiont strains, which would negatively affect the host and could promote the evolution of symbiotic cheaters (Bronstein et al., 2003; Denison, 2000; Frank, 1996; Noë

and Hammerstein, 1994; Sachs et al., 2004; Trivers, 1971; West et al., 2002; Wilkinson and Sherratt, 2001). In such situations, natural selection might favor the evolution of symbiont discrimination and/or policing systems in the host organism. In the legume–*Rhizobium* and squid–*Vibrio* symbioses, infection capability known as “host range” (or “symbiont range”) is governed by specific host–symbiont molecular cross-talk, mediated by bacterial surface peptideglycans and lipopolysaccharides (Cloud-Hansen et al., 2006; Nyholm and McFall-Ngai, 2004; Perret et al., 2000). An experimental study by using *Rhizobium* mutant strains deficient in nitrogen-fixing ability demonstrated a host sanction mechanism in the legume–*Rhizobium* symbiosis, wherein leguminous plants punish low-performers by decreasing oxygen levels within the parasitized nodules (Kiers et al., 2003). Genetic diversity of the microbial partners, however, may not always be bad for the host. Several theoretical studies have suggested that the symbiont heterogeneity could provide the host with an opportunity to choose symbiont types with desirable properties, which might be advantageous for the host under fluctuating environmental conditions (Noë and Hammerstein, 1994, 1995; Wilkinson and Sherratt, 2001). One of the clear-cut cases was reported in the coral symbiosis. Individual corals commonly carry multiple dinoflagellate strains, each of which exhibits different performance under different photo and thermal conditions, and the host corals change the symbiont compositions seasonally to cope with the heterogeneous oceanic environment (Rowan, 1998; Rowan et al., 1997). From this point of view, the genetic diversity observed in the *Burkholderia* symbiont (0–4% sequence diversity in 16S rRNA gene; Figure 7.4) might be meaningful. In this context, it is of interest to examine whether the individual *Burkholderia* symbiont strains exhibit different biological capabilities under different environmental conditions.

Concluding remarks and perspectives

The novel endosymbiotic system found in the alydid stinkbugs possibly provides a unique opportunity for studying insect–bacteria mutualisms. In several symbiotic systems with environmental transmission such as legume–*Rhizobium* and squid–*Vibrio* associations, because the bacterial symbionts are culturable and genetically manipulatable, these model systems have greatly contributed to our knowledge of the molecular mechanisms underlying the host–symbiont interactions, such as nutritional exchanges, host–symbiont specificity, development of symbiotic organs, etc. (reviewed in Gualtieri and Bisseling, 2000; Perret et al., 2000). In contrast, only a small number of insect symbionts have been successfully cultured in cell-free media (Beard et al., 1992; Dale et al., 2006; Dale and Maudlin, 1999; Gherna et al., 1991; Hackett et al., 1986), and most of them are not beneficial but rather parasitic ones (Pontes and Dale, 2006). In the alydid–*Burkholderia* system, the host insect is easily maintainable in the laboratory, and the symbiont is easily culturable in standard microbiological media (Figure 7.5). Considering that several transposon-mediated transformation systems have already been established in other *Burkholderia* species (Choi et al., 2006; DeShazer et al., 1997; Hunt et al., 2004; Lefebvre and Valvano, 2002), the symbiont is potentially manipulatable genetically. Reintroduction of genetically manipulated symbiont into the host stinkbug could provide unique opportunities to investigate bacterial genes responsible for the host–symbiont interactions. The alydid–*Burkholderia* symbiosis would provide a novel system that enables molecular genetic approaches to the mechanisms underlying the insect–microbe mutualisms.

Upon the recent establishment of the experimental system for the alydid–*Burkholderia* symbiosis, we now have a number of intriguing questions to be addressed in future studies, as listed below:

1. Do the host and symbiont coadapt to each other? Is there any host-specificity of the symbiont? The phylogeny of the *Burkholderia* symbiont isolates exhibited no correlation with the host taxonomy and geographic distribution (Figure 7.4), leading to the hypothesis that the host insects and the symbiotic bacteria do not coadapt to each other and there is no strict "host range" of the symbionts. The hypothesis is testable by experimental reciprocal exchanges of the symbiont strains within and between the stinkbug species.
2. How prevalent is the *Burkholderia* symbiont in the Heteroptera? A number of heteropterans possess midgut crypts and harbor specific bacteria inside, only a few of which have been microbiologically characterized thus far. Broad survey of the *Burkholderia* infection will unveil prevalence of the symbiont in the Heteroptera. Subsequent phylogenetic analyses of both the symbiotic bacteria and the host insects would shed light on the evolutionary process of the unique relationships.
3. What mechanisms ensure the specific and efficient infection of the *Burkholderia* symbiont? Because an enormous microbial diversity exists in the soil environment (Garbeva et al., 2004), the *Burkholderia* symbiont must constitute only a very small fraction of the microbes that are ingested by the stinkbug nymphs. In the legume-*Rhizobium* and the squid-*Vibrio* symbioses, intricate cellular and molecular host-symbiont cross-talks mediated by bacterial surface peptidoglycans and lipopolysaccharides are shown to involve the establishment of selective symbiosis (Freiberg et al., 1997; Nyholm and McFall-Ngai, 2004; Oldroyd and Downie, 2004; Perret et al., 2000). Such molecules might play an important role in the alydid-*Burkholderia* symbiosis, which could be investigated by genetic approaches in future studies.
4. What function does the symbiont play for the host stinkbug? Members of the genus *Burkholderia* are major soil bacteria that are most commonly found in plant rhizosphere (Coenye and Vandamme, 2007; Woods and Sokol, 2000), and show a wide variety of biological activities: a number of strains possess nitrogen-fixing ability (Estrada-De et al., 2001); some strains nodulate the root of leguminous plants where the bacteria fix atmospheric dinitrogen (Chen et al., 2003; Moulin et al., 2001); some species are associated with plant leaf galls (Van Oevelen et al., 2002; Van Oevelen et al., 2004); some members promote plant growth and suppress plant diseases (Bevivino et al., 1998) and are used as biofertilizing agents (Vessey, 2003). Such biological activities of the bacterial group might be relevant to biological functions of the *Burkholderia* symbiont in the host stinkbugs, constituting challenging targets of future studies.

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Mutualism revealed by symbiont genomics and bacteriocyte transcriptomics

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Introduction

Many insect lineages, especially members of Sternorrhyncha (aphids, psyllids, whiteflies, and scale insects), Auchenorrhyncha (cicadas, leafhoppers, treehoppers, spittlebugs, and planthoppers), Blattaria (cockroaches), and Coleoptera (beetles), have bacteriocytes (also called mycetocytes), cells that are differentiated to harbor obligate mutualistic intracellular bacteria (Buchner, 1965). The bacteria, usually called primary symbionts, are confined to the cytoplasm of bacteriocytes except during transmissions to eggs or progeny and have been vertically transmitted through host generations for hundreds of millions of years (Moran et al., 1993; Chen et al., 1999; Thao et al., 2000; Lo et al., 2003; Thao and Baumann, 2004; Baumann and Baumann, 2005; Moran et al., 2005b; Takiya et al., 2006; Gruwell et al., 2007). The host insects and the primary symbionts are indispensable to each other for their growth and reproduction: the symbionts cannot proliferate

out of bacteriocytes, whereas the host insects grow poorly and are sterile when they are deprived of symbionts (Douglas, 1989). Such indivisibility is reminiscent of the association between extant eukaryotic cells and organelles such as mitochondria and chloroplasts, which are now widely acknowledged to be descendants of free-living bacteria that invaded into ancient ancestors of eukaryotes far more than one billion years ago (Margulis, 1970; Dyall et al., 2004; Poole and Penny, 2007).

Buchner (1965) advocated that bacteriocyte symbioses have evolved multiple times between various insect groups and a diverse array of bacteria, and this outlook was verified by molecular phylogenetic analyses. The primary symbionts of aphids (*Buchnera aphidicola*), psyllids (*Carsonella ruddii*), whiteflies (*Portiera aleyrodidarum*), some leafhopper species (*Baumannia cicadellinicola*), tsetse flies (*Wigglesworthia glossinidia*), and ants (*Blochmannia*) belong to γ -Proteobacteria (Munson et al., 1991; Chen et al., 1999; Thao et al., 2000; Moran et al., 2003; Thao and Baumann, 2004), whereas those of manyuchenorrhynchan insects (*Sulcia muelleri*) and armored scale insects belong to Bacteroidetes (Moran et al., 2005b; Gruwell et al., 2007). A fascinating case was exemplified in mealybugs, where β -proteobacterial primary symbionts (*Tremblaya princeps*) contain γ -proteobacterial “secondary” symbionts (von Dohlen et al., 2001). These endosymbionts of different host groups have evolved as independent lineages from free-living bacteria. In general, primary symbionts appear to have a nutritional role, as the host insects in many cases feed on very specialized diets such as plant sap (Auchenorrhyncha and Sternorrhyncha) or blood (tsetse flies), which are poor in essential amino acids (amino acids that metazoa cannot synthesize: tryptophan, lysine, methionine, phenylalanine, threonine, valine, leucine, isoleucine, arginine, and histidine) and B-complex vitamins, respectively (Douglas, 1989; Sandstrom and Moran, 1999). Physiological studies have corroborated that *Buchnera* and *Wigglesworthia* provide hosts with these nutrients (Nogge, 1981; Sasaki and Ishikawa, 1995; Douglas, 1998).

Although the inseparability of two partners has been the core interest, it also limited investigations of the host–symbiont interactions. A breakthrough was brought about by the advent of genomics and transcriptomics, which enable us to obtain comprehensive genetic information on the bacteriocyte symbioses. In this chapter, I review the genomics of bacteriocyte-restricted primary symbionts and the transcriptomics of the host bacteriocytes, featuring the smallest cellular genome of *Carsonella*, and the transcriptome analysis of the aphid bacteriocyte that harbors *Buchnera*.

General features of the genomes of bacteriocyte symbionts

By the time the whole genome sequence of *Carsonella* was determined, several genomes of primary symbionts that are associated with insect bacteriocytes had been sequenced, including four strains of *Buchnera* from aphids (those in association with *Acyrtosiphon pisum*, *Schizaphis graminum*, *Baizongia pistaciae*, and *Cinara cedri*, which are called *Buchnera*-APS, *Buchnera*-Sg, *Buchnera*-Bp, and *Buchnera*-Cc, respectively), *Wigglesworthia* from tsetse flies, two *Blochmannia* species (*Bl. floridanus* and *Bl. pennsylvanicus*) from carpenter ants, and *Baumannia* from sharpshooters (Shigenobu et al., 2000; Tamas et al., 2002; van Ham et al., 2003; Pérez-Brocal et al., 2006; Akman et al., 2002; Gil et al., 2003; Degnan et al., 2005; Wu et al., 2006). These genomes showed a distinctive set of traits as follows:

1. *Reduced size*: The genomes range from 0.42 to 0.79 megabase in size and contain about 400–700 genes (Table 8.1), whereas related bacteria such as those in the *Enterobacteriaceae* within the γ -Proteobacteria have much larger genomes (3–6 megabases encoding 3,000 to 6,000 genes) (NCBI Entrez Genome/Bacteria, http://www.ncbi.nlm.nih.gov/genomes/static/eub_g.html). These genomes are derived from ancestral genomes with typical sizes, through a process of gene inactivation and deletion (Mira et al., 2001; Moran, 2003).
2. *Low G + C content*: The G + C content of the genomes ranges from 20% to 33% (Table 8.1). In bacteria, the value appears to be correlated to the genome size, and the force to increase A + T seems to reflect the loss of genes for DNA repair enzymes, or decreased efficacy of these enzymes. For example, misincorporation of dUTP during DNA replication and spontaneous C→U deamination will create an A + T-biased mutational pressure, if not prevented or corrected by appropriate enzymes (Glass et al., 2000).
3. *Fast polypeptide evolution*: The amino acid substitution rate is significantly higher than those in free-living relatives. The rate for ribosomal RNA genes is also elevated, and this accelerated evolution is evident in numerous bacteriocyte associates for which only rRNA and not the whole genome is sequenced (Moran, 1996; Ochman et al., 1999).

These features appear to be related to one another and are observed in a variety of obligately host-associated bacteria of both symbiotic and parasitic nature (Andersson et al., 1998; Stephens et al., 1998; Oshima et al., 2004; Wu et al., 2004). In bacteria, there seems to be a constant eroding force of nucleotide deletion that must be counterbalanced by selection on gene function (Mira et al., 2001), which means that reduced efficacy of selection leads to genome reduction (Moran, 2003). Two different factors that reduce the selection efficiency are proposed. First, as the obligate parasites and symbionts can depend on the constant supply of metabolites in the stable host milieu, the pressure to maintain genes for redundant and unnecessary pathways would be eliminated. Second, as the small effective population sizes of these bacteria lead to higher levels of fixation of slightly deleterious mutations, eroding forces of mutation and genetic drift can be increased even when the intensity of selection does not change, which results in loss of beneficial but nonessential genes (Moran, 2003). Every incidence of the loss of DNA repair genes would further increase the evolutionary speed, mutational bias toward A + T, and the deleterious mutations fixed in the population, leading to the reduced genomes.

Despite loss of many ancestral genes, the genomes of insect primary symbionts retain numerous genes that underpin biosynthetic pathways supplying molecules needed for host nutrition. Inconsistent with physiological data, *Buchnera* and *Wigglesworthia* retain genes for biosynthesis of essential amino acids and B-complex vitamins, respectively (Shigenobu et al., 2000; Tamas et al., 2002; van Ham et al., 2003; Pérez-Brocal et al., 2006; Akman et al., 2002). Whereas genome reduction is generally observed in obligately host-associated bacteria of both parasitic and mutualistic nature, the genomes of the parasites lack most genes for synthesis of such nutrients (Andersson et al., 1998; Stephens et al., 1998; Oshima et al., 2004). Thus, the gene repertoires of the genomes of primary symbionts in insects clearly reflect their mutualistic nature for the host insects. Such genes that increase host fitness should have been retained under strong selective pressure.

Also, these bacteriocyte associates are among numerous host-dependent lineages that have been considered as representing near-minimal genomes. However, all cases of genome reduction appeared to reach a limit of about 400 kb and about 20% G + C, which

Table 8.1 Comparison of Genomes of Bacteriocyte Symbionts in Insects

Feature	Carsonella	Buchnera- APS	Buchnera-Sg	Buchnera-Bp	Buchnera-Cc	Wiggles- worthia	Bl. floridanus	Bl. pennsylvanicus	Baumannia	Sulcia
Chromosome (bp)	159,662	640,681	641,454	615,980	416,380	697,724	705,557	791,654	686,194	245,530
Plasmid (bp)	0	2 (15,044)	2 (11,547)	1 (2,399)	1 (6,054)	1 (5,280)	0	0	0	0
G + C content (%)	16.5	26.2	25.3	25.3	20.1	22.5	27.4	29.6	33.2	22.4
Predicted ORFs (no. on plasmids in parenthesis)	182	572 (9)	554 (9)	507 (3)	357 (5)	617 (6)	589	610	605	228
Average length of ORFs (bp)	826	987	981	992	994	990	1,006	995	982	1,006
tRNAs	28	32	32	32	31	34	37	39	39	31
rRNAs	3	3	3	3	3	6	3	3	6	3
Protein and RNA coding regions (%)	97.3	88.1	84.7	82.4	86.9	88.5	84.3	77.7	88.5	96.1

used to be believed to be the minimal limits for cellular organism. This premise was abolished by the whole genome analysis of *Carsonella* (Nakabachi et al., 2006).

The smallest cellular genome

Carsonella ruddii

Carsonella (Figure 8.1) is the primary symbiont that appears to be present in all psyllids (superfamily Psylloidea), a group of about 2,500 insect species that feed on plant phloem sap (Thao et al., 2000; Spaulding and von Dohlen, 2001; Gullan and Martin, 2003). Phylogenetic analyses based on rRNA genes indicate that *Carsonella* is an independent lineage within the γ -Proteobacteria falling outside of the *Enterobacteriaceae* to which many other endosymbionts, including *Buchnera*, *Baumannia*, *Blochmannia*, and *Wigglesworthia*, belong (Thao et al., 2000; Spaulding and von Dohlen, 1998; Fukatsu and Nikoh, 1998). The phylogenetic congruence of psyllids and *Carsonella* supports an origin of the symbiosis prior to the diversification of living psyllids, about 250 million years ago, which is estimated based on insect fossils (Thao et al., 2000; Spaulding and von Dohlen, 2001). Because psyllids are closely related to aphids and, like aphids, feed on phloem sap, *Carsonella* is also believed to provide host psyllids with essential amino acids (Thao et al., 2000; Spaulding and von Dohlen, 2001). Clark et al. (2001) sequenced three DNA fragments (37 kb in total) of *Carsonella* and found that they have unusual properties, including exceptionally low GC content (19.9%), absence of intergenic spacers, operon fusion, and the lack of the complement of the Shine-Dalgarno (SD) sequence at the 3' end of 16S rDNA, implying that the *Carsonella*

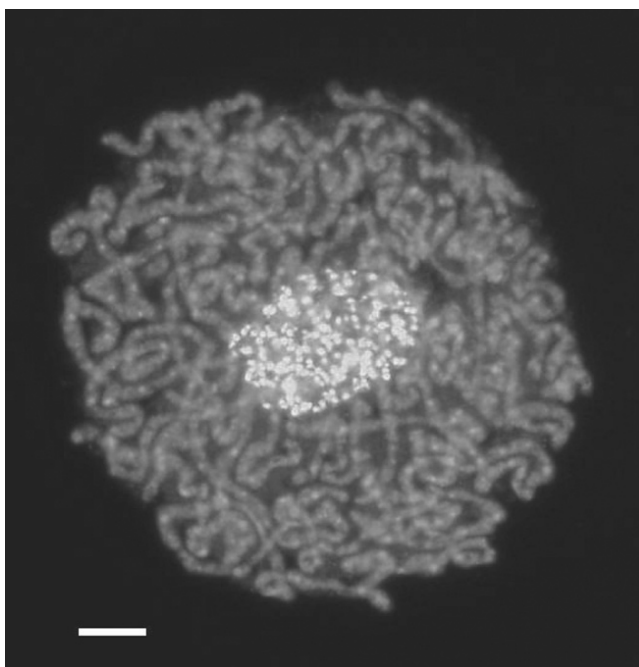


Figure 8.1 (Color figure follows p. 238.) The bacteriocyte of *P. venusta*. Tubular cells surrounding the host nucleus (center) are *Carsonella*. Bar = 10 μ m. (Modified from Nakabachi, A., Yamashita, A., Toh, H., Ishikawa, H., Dunbar, H.E., Moran, N.A., and Hattori, M. [2006]. *Science* **314**: 267.)

genome had undergone unique degenerative processes. Inspired by this preceding work, the whole genome sequence of *Carsonella* was analyzed (Nakabachi et al., 2006). The hackberry petiole gall psyllid *Pachypsylla venusta* was used for the analysis, because this species has no other microbial symbionts that would potentially disturb the analysis (Spaulding and von Dohlen, 1998; Thao et al., 2000; Spaulding and von Dohlen, 2001). The absence of additional symbionts has been confirmed with several methods, including PCR, Southern hybridization, sequencing of random clones, and microscopy.

Streamlining gone too far

The complete genome sequence of *Carsonella* of *P. venusta* (*Carsonella*-Pv) revealed that it is indeed an exceptional genome, far smaller and more biased in base composition than any reported cellular genome (Table 8.1; Nakabachi et al., 2006). The genome is a single circular chromosome of 159,662 base pairs (bp) with no plasmids. The genome is only about one third the size of that of the *Buchnera*-Cc strain (previously known as the smallest bacterial genome, at about 420 kb; Pérez-Brocail et al., 2006), and is approximately equivalent to that of chloroplast genomes (generally 120–200 kb; NCBI Entrez Genome/Eukaryota/Organelles, http://www.ncbi.nlm.nih.gov/genomes/static/euk_o.html). Besides small size, AT-richness is a typical genomic feature of primary symbionts as mentioned in the previous section, and *Carsonella*-Pv is again the extreme case. The GC content of the *Carsonella* genome averages only 16.5%, considerably lower than that of any known cellular genome. The average GC content of ORFs alone is 15.9%, and the inferred proteins of *Carsonella*-Pv are biased strongly toward amino acids that are encoded by AT-rich codons.

Gene prediction and annotation identified 182 open reading frames (ORFs) with an average length of 826 bp. Putative functions were assigned to 136 (74.7%) of the ORFs. Only four ORFs (2.2%) appeared to retain the canonical SD sequence, consistent with previous findings (Clark et al., 2001). The chromosome encodes one copy of a 16S-23S-5S ribosomal RNA operon and 28 transfer RNA genes for all 20 amino acids. No genes related to phage, transposons, or insertion sequences are found in the genome. In the genome, 44 of 182 ORFs correspond to hypothetical proteins with no recognizable homologues in databases; this proportion is much higher than that observed in other sequenced genomes of primary symbionts, which possess few novel genes. These ORFs, whose average GC content is 10.3%, may represent rapidly evolving genes that have lost detectable homology. These hypothetical proteins appear to be real, because stop codons occur with high frequency in AT-rich sequences that are not under purifying selection. At the observed base composition, random sequences would give an average ORF length of under 10 codons, and a 150 base pair random sequence (potentially encoding 50 amino acids) has less than a 1% chance of not being truncated by a stop codon.

The genome shows a substantial reduction in the length of ORFs. The average length of *Carsonella* ORFs that are conserved among insect primary symbionts is 908 bp, which is 17.8%–18.4% shorter than those of orthologs (Nakabachi et al., 2006). Although no direct evidence is available regarding the integrity of function of these shortened ORFs, their near-typical length implies that most are preserved by purifying selection and therefore are still functional.

Another remarkable feature in this genome is an extremely high gene density. The protein-coding sequences and RNA genes cover 97.3% of the whole genome (Table 8.1). This high density is attributed to numerous overlapping genes and few intergenic spacers. Out of 182 ORFs, 164 (90%) overlap with at least one of the two adjacent ORFs and the average length of all 132 overlapping regions is 10.7 nt, ranging from 1 to 49 nt. The

majority (92%) are tandem overlaps that occur on the same strand, and in all cases, genes overlap out of frame. *Carsonella* has only 30 intergenic spacers, whereas even other primary symbionts have a typical length of intergenic spacers between almost all adjacent genes (Nakabachi et al., 2006). Thus, it is feasible to say that the extremely streamlined genome of *Carsonella*-Pv consists of (1) *small number* of (2) *short* (3) *overlapped* ORFs.

Mutualistic history carved in the genome

Classification of ORFs based on clusters of orthologous groups (COG) indicates that 52.2% of the total ORFs in the *Carsonella*-Pv genome are devoted to two COG functional categories, translation (J, 34.6%) and amino acid metabolism (E, 17.6%) (Nakabachi et al., 2006). In the latter category, *Carsonella*-Pv retains many genes for biosynthesis of essential amino acids but few genes for biosynthesis of nonessential amino acids, as in the genomes of *Buchnera*, the primary symbiont of aphids (Shigenobu et al., 2000; Tamas et al., 2002; van Ham et al., 2003; Pérez-Brocal et al., 2006). As both psyllids and aphids feed only on plant phloem sap that is poor in essential amino acids, the analogy of gene repertoires in *Carsonella* and *Buchnera* is an intriguing example of convergence. The retention of a number of genes involved in essential amino acid biosynthesis in the extremely small *Carsonella* genome clearly reflects an evolutionary history of selection for retaining these genes due to their mutualistic effects in providing essential amino acids to the host insects. However, further study is required to determine whether extant *Carsonella* is truly able to synthesize essential amino acids, as the pathways appear to be partially incomplete. As nymphs of *P. venusta* grow within closed galls that are believed to be nutrient sinks (this was verified in several cases; Larson and Whitham, 1991; Inbar et al., 1995; Fay et al., 1996), phloem sap in *P. venusta* galls might have enough essential amino acids, and *Carsonella*-Pv could have lost some of the pathways to synthesize essential amino acids. It will be of interest to explore the *Carsonella* genome in other psyllid species, which do not induce galls or conspicuous plant damage.

Extremely poor gene repertoire

Another remarkable feature of the genome is the complete loss of genes in many COG categories (Table 8.2). No genes for categories of nucleotide metabolism (F), lipid metabolism (I), coenzyme metabolism (H), defense mechanism (V), signal transduction (T), cell motility (N), cell envelope biogenesis (M), and intracellular trafficking (U), representing a half of all COG categories, were found (Nakabachi et al., 2006), whereas other endosymbionts were demonstrated to have genes in almost all COG categories (Shigenobu et al., 2000; Tamas et al., 2002; Akman et al., 2002; van Ham et al., 2003; Gil et al., 2003; Degnan et al., 2005; Wu et al., 2006; Pérez-Brocal et al., 2006).

Carsonella-Pv completely lacks genes for biosynthesis of fatty acid, phospholipid, lipopolysaccharide, and peptidoglycan. The absence of these genes suggests that *Carsonella* is unable to synthesize its cell membrane and cell wall by their own mechanisms, whereas, of course, electron micrographs show the presence of cell envelopes (Chang and Musgrave, 1969; Waku and Endo, 1987; Thao et al., 2000). The genome also lacks genes for cell division, such as *minCDE*, *ftsAZ*, and *ftsW*. The *mreBCD* genes encoding cell-shape-determining proteins are missing as well. Although the *Carsonella* cell is very elongated in shape in maternal bacteriocytes (Figure 8.1; Buchner, 1965; Chang and Musgrave, 1969; Nakabachi et al., 2006), they need to divide to make short “infectious forms” before transmission to

Table 8.2 Genes Predicted in the *Carsonella*-Pv Genome

ID	Start	Stop	Direction	Protein	COG	Gene
CR001	1	1317	+	tRNA_modification_GTPase	R	<i>thdF</i>
CR002	1314	2816	+	glucose_inhibited_division_protein_A	D	<i>gidA</i>
CR003	2785	3477	+	F0F1-type_ATP_synthase_A_subunit	C	<i>atpB</i>
CR004	3486	3719	+	F0F1-type_ATP_synthase_C_subunit	C	<i>atpE</i>
CR005	3721	4176	+	putative_F0F1-type_ATP_synthase_B_subunit	C	<i>atpF</i>
CR006	4169	4354	+	hypothetical_protein		
CR007	4344	5789	+	F0F1-type_ATP_synthase_alpha_subunit	C	<i>atpA</i>
CR008	5786	6544	+	F0F1-type_ATP_synthase_gamma_subunit	C	<i>atpG</i>
CR009	6541	7884	+	F0F1-type_ATP_synthase_beta_subunit	C	<i>atpD</i>
CR010	7881	8123	+	hypothetical_protein		
CR011	8110	8997	+	ornithine_carbamoyltransferase	E	<i>argF</i>
CR012	9521	9084	-	3-dehydroquinate_dehydratase	E	<i>aroD</i>
CR013	11407	9497	-	transketolase	G	<i>tktB</i>
CR014	11394	13061	+	hypothetical_protein		
CR015	13048	13767	+	5,10-methylenetetrahydrofolate_reductase	E	<i>metF</i>
CR016	13892	13755	-	hypothetical_protein		
tRNA-Ile	14011	14084	+			
CR017	15115	14072	-	succinyl-diaminopimelate_desuccinylase	E	<i>dapE</i>
CR018	16080	15115	-	tetrahydrodipicolinate_N-succinyltransferase	E	<i>dapD</i>
CR019	16784	16074	-	methionine_aminopeptidase	J	<i>map</i>
CR020	16770	17420	+	ribosomal_protein_S2	J	<i>rpsB</i>
CR021	17414	18040	+	hypothetical_protein		
CR022	18033	18563	+	hypothetical_protein		
CR023	18556	21897	+	DNA_polymerase_III_alpha_subunit	L	<i>dnaE</i>
CR024	21894	22382	+	hypothetical_protein		

CR025	22428	24530	+	5-methyltetrahydropteroyltriglutamate—homocysteine_S-methyltransferase	E	<i>metE</i>
CR026	24669	24857	+	ribosomal_protein_L31	J	<i>rpmE</i>
CR027	24835	25293	+	hypothetical_protein	E	
CR028	25274	26320	+	3-dehydroquinase_synthase	E	<i>aroB</i>
CR029	26274	27923	+	dihydroxy-acid_dehydratase	E	<i>ilvD</i>
CR030	27916	29010	+	conserved_hypothetical_protein		
CR031	29000	30196	+	serine_hydroxymethyltransferase	E	<i>glyA</i>
CR032	30317	31666	+	ABC_transporter_permease_component	O	
CR033	31663	32328	+	ABC_transporter_ATP-binding_component	O	
CR034	32319	33146	+	hypothetical_protein		
CR035	33143	34297	+	selenocysteine_lyase	E	<i>yfiO</i>
CR036	35155	34259	-	putative_tRNA(5-methylaminomethyl-2-thiouridylate)_methyltransferase	J	<i>trnLU</i>
CR037	35379	35155	-	translation_initiation_factor_IF-1	J	<i>infA</i>
tRNA-Glu	35392	35462	+			
tRNA-Gly	35463	35534	+			
tRNA-Ser	35535	35619	+			
CR038	35914	35618	-	putative_thioredoxin	O	
CR039	37212	35911	-	6-phosphogluconate_dehydrogenase	G	<i>gnd</i>
tRNA-Asn	37565	37637	+			
tRNA-Met	37710	37638	-			
CR040	38177	37719	-	hypothetical_protein		
CR041	38340	39029	+	putative_peptide_chain_release_factor_A	J	<i>prfA</i>
CR042	40130	39033	-	aspartyl/glutamyl-tRNA_amidotransferase_B_subunit	J	<i>gatB</i>
CR043	41478	40117	-	aspartyl/glutamyl-tRNA_amidotransferase_A_subunit	J	<i>gatA</i>
CR044	41675	41475	-	hypothetical_protein		
CR045	41767	42060	+	ribosomal_protein_L13	J	<i>rplM</i>
CR046	42057	42437	+	ribosomal_protein_S9	J	<i>rpsL</i>

Continued.

Table 8.2 Genes Predicted in the *Carsonella*-Pv Genome (Continued)

ID	Start	Stop	Direction	Protein	COG	Gene
CR047	42558	43139	+	hypothetical_protein		
CR048	43129	43887	+	hypothetical_protein		
CR049	43884	45107	+	3-phosphoshikimate_1-carboxyvinyltransferase	E	<i>aroA</i>
CR050	45091	46197	+	putative_ribosomal_protein_S1	J	<i>rpsA</i>
CR051	47786	46194	-	chaperonin_GroEL	O	<i>mopA</i>
CR052	48069	47776	-	chaperonin_GroES	O	<i>mopB</i>
tRNA-Val	48215	48287	+			
tRNA-Asp	48296	48369	+			
CR053	48573	48971	+	hypothetical_protein		
CR054	48961	49422	+	hypothetical_protein		
CR055	49416	50561	+	putative_replicative_DNA_helicase	L	<i>dnaB</i>
CR056	50533	51102	+	hypothetical_protein		
CR057	51080	51217	+	hypothetical_protein		
CR058	51214	51987	+	putative_DNA_primase	L	<i>dnaG</i>
CR059	51984	52946	+	putative_RNA_polymerase_sigma_factor_rpoD	K	<i>rpoD</i>
tRNA-Ala	52958	53029	+			
tRNA-Phe	53154	53226	+			
tRNA-Gln	53227	53298	+			
tRNA-Thr	53302	53373	+			
CR060	53892	53542	-	hypothetical_protein		
CR061	53898	54353	+	chaperone_protein_GrpE	O	<i>grpE</i>
CR062	54408	56216	+	chaperone_protein_DnaK	O	<i>dnaK</i>
CR063	56273	56587	+	hypothetical_protein		
CR064	56580	57278	+	dihydrodipicolinate_reductase	E	<i>dapB</i>
CR065	57272	57721	+	truncated_carbamoylphosphate_synthase_small_subunit	E	<i>carA</i>

CR066	57714	58253	+	truncated_carbamoylphosphate_synthase_small_subunit	E	<i>carA</i>
CR067	58246	61143	+	carbamoylphosphate_synthase_large_subunit	E	<i>carB</i>
tRNA-Leu	61144	61216	+			
CR068	61235	62845	+	putative_translation_initiation_factor_IF-2	J	<i>infB</i>
CR069	62817	63014	+	hypothetical_protein		
CR070	63011	63988	+	tyrosyl-tRNA_synthetase	J	<i>tyrS</i>
CR071	63948	65123	+	glutaminyl-tRNA_synthetase	J	<i>glrS</i>
tRNA-Pro	65163	65236	+			
tRNA-Arg	65237	65310	+			
tRNA-His	65313	65385	+			
CR072	65412	65996	+	ATP-dependent_Clp_protease_proteolytic_subunit	O	<i>clpP</i>
CR073	65971	67038	+	ATP-dependent_Clp_protease_ATP-binding_subunit	O	<i>clpX</i>
CR074	67801	68127	+	hypothetical_protein		
CR075	68124	69401	+	aspartyl-tRNA_synthetase	J	<i>aspS</i>
tRNA-Ser	69415	69499	+			
CR076	69553	70074	+	ribulose-phosphate_3-epimerase	G	<i>rpe</i>
CR077	73120	70076	-	delta-1-pyrroline-5-carboxylate_dehydrogenase	C	<i>putA</i>
CR078	73136	74479	+	2-isopropylmalate_synthase	E	<i>leuA</i>
CR079	74472	75509	+	chorismate_synthase	E	<i>aroC</i>
CR080	75502	76872	+	3-isopropylmalate_dehydratase_large_subunit	E	<i>leuC</i>
CR081	76856	77422	+	3-isopropylmalate_dehydratase_small_subunit	E	<i>leuD</i>
CR082	77419	78468	+	3-isopropylmalate_dehydrogenase	C	<i>leuB</i>
CR083	78461	79486	+	aspartate-semialdehyde_dehydrogenase	E	<i>asd</i>
CR084	79497	80507	+	seryl-tRNA_synthetase	J	<i>serS</i>
tRNA-Leu	80487	80565	+			
CR085	80599	80970	+	hypothetical_protein		
CR086	81565	81029	-	hypothetical_protein		

Continued.

Table 8.2 Genes Predicted in the *Carsonella-Pv* Genome (Continued)

ID	Start	Stop	Direction	Protein	COG	Gene
CR087	82107	81562	-	DNA_polymerase_III_epsilon_subunit	L	<i>dnaQ</i>
CR088	83321	82104	-	hypothetical_protein		
CR089	83577	83314	-	putative_phenylalanyl-tRNA_synthetase_alpha_subunit	J	<i>pheS</i>
CR090	84284	83979	-	ribosomal_protein_L20	J	<i>rplT</i>
CR091	84926	84402	-	translation_initiation_factor_IF-3	J	<i>infC</i>
CR092	85534	84926	-	superoxide_dismutase	P	<i>sodA</i>
CR093	86044	85571	-	hypothetical_protein		
CR094	86949	86041	-	3-deoxy-7-phosphoheptulonate_synthase	E	<i>aroH</i>
CR095	87068	86937	-	hypothetical_protein		
CR096	87069	88349	+	conserved_hypothetical_protein	J	<i>yleA</i>
CR097	88324	88617	+	hypothetical_protein		
CR098	88604	90502	+	leucyl-tRNA_synthetase	J	<i>leuS</i>
CR099	91316	90492	-	succinyl-CoA_synthetase_alpha_subunit	C	<i>sucD</i>
CR100	92350	91313	-	succinyl-CoA_synthetase_beta_subunit	C	<i>sucC</i>
CR101	93345	92347	-	putative_glutamyl-tRNA_synthetase	J	<i>gltX</i>
tRNA-Met	93431	93359	-			
CR102	93518	94816	+	methionyl-tRNA_synthetase	J	<i>metG</i>
CR103	94806	94991	+	hypothetical_protein		
CR104	96165	94984	-	argininosuccinate_synthase	E	<i>argG</i>
tRNA-Cys	96209	96279	+			
tRNA-Leu	96280	96361	+			
CR105	97107	96343	-	dihydrodipicolinate_synthase	E	<i>dapA</i>
tRNA-Lys	97213	97140	-			
CR106	97515	97204	-	hypothetical_protein		
CR107	97509	98876	+	malate:quinone_oxidoreductase	R	<i>mgo</i>

Table 8.2 Genes Predicted in the *Carsonella*-Pv Genome (Continued)

ID	Start	Stop	Direction	Protein	COG	Gene
CR129	122787	120313	-	isoleucyl-tRNA_synthetase	J	<i>ileS</i>
CR130	122923	122777	-	hypothetical_protein		
CR131	123672	122908	-	putative_GTPase	R	<i>yhbZ</i>
CR132	123916	123662	-	ribosomal_protein_L27	J	<i>rpmA</i>
CR133	124227	123919	-	hypothetical_protein		
CR134	124460	124224	-	hypothetical_protein		
CR135	125418	124450	-	RNA_polymerase_alpha_subunit	K	<i>rpoA</i>
CR136	126008	125415	-	ribosomal_protein_S4	J	<i>rpsD</i>
CR137	126361	126005	-	ribosomal_protein_S11	J	<i>rpsK</i>
CR138	126704	126348	-	ribosomal_protein_S13	J	<i>rpsM</i>
CR139	126814	126701	-	ribosomal_protein_L36	J	<i>rpmJ</i>
CR140	127047	126811	-	putative_ribosomal_protein_L15	J	<i>rplO</i>
CR141	127466	127047	-	ribosomal_protein_S5	J	<i>rpsE</i>
CR142	127674	127438	-	hypothetical_protein		
CR143	128216	127671	-	ribosomal_protein_L6	J	<i>rplF</i>
CR144	128539	128168	-	ribosomal_protein_S8	J	<i>rpsH</i>
CR145	128820	128533	-	ribosomal_protein_S14	J	<i>rpsN</i>
CR146	129322	128822	-	ribosomal_protein_L5	J	<i>rplE</i>
CR147	129687	129319	-	ribosomal_protein_L14	J	<i>rplN</i>
CR148	129935	129684	-	ribosomal_protein_S17	J	<i>rpsQ</i>
CR149	130335	129928	-	ribosomal_protein_L16	J	<i>rplP</i>
CR150	130936	130325	-	ribosomal_protein_S3	J	<i>rpsC</i>
CR151	131234	130920	-	putative_ribosomal_protein_L22	J	<i>rplV</i>
CR152	131497	131231	-	ribosomal_protein_S19	J	<i>rpsS</i>
CR153	132201	131494	-	ribosomal_protein_L2	J	<i>rplB</i>

Table 8.2 Genes Predicted in the *Carsonella-Pv* Genome (Continued)

ID	Start	Stop	Direction	Protein	COG	Gene
CR178	156402	157157	+	diaminopimelate_epimerase	E	dapF
CR179	157154	157897	+	glycyl-tRNA_synthetase_alpha_subunit	J	glyQ
CR180	157894	158382	+	hypothetical_protein		
CR181	158603	158400	-	cold_shock_protein	K	cspE
CR182	159662	158649	-	hypothetical_protein		

oocytes (Buchner, 1965; Waku and Endo, 1987), raising a question as to how they achieve it with the complete lack of genes for cell division.

All known genes necessary for glycolysis and tricarboxylic acid (TCA) cycle are missing except those encoding succinyl-CoA synthetase subunits (CR099 and CR100) (Table 8.2). In *Carsonella*-Pv, six genes (CR003–CR005, CR007–CR009) for F_0F_1 type ATP synthase subunits (except delta and epsilon) are conserved with the same gene organization as in other related bacteria, whereas *ydiC*, which seems essential for proper integration of the ATP synthase into the membrane, is absent. Four genes (CR167–CR170) encoding cytochrome O ubiquinol oxidase subunits are identified, but the genes for ubiquinone biosynthesis are missing in *Carsonella*. Further studies are required to know whether *Carsonella* can produce ATP by carrying out oxidative respirations or whether it imports ATP from its host. In general, it appears that bacteriocyte-restricted primary symbionts are unable to synthesize many of the essential metabolites and, thus, must rely on the host bacteriocyte to obtain them (Zientz et al., 2004; Nakabachi et al., 2005). Whereas *Carsonella*-Pv has the most limited biosynthetic capacities as mentioned above, the genome encodes only a single transporter (ABC transporter), which consists of a permease (CR032) and an ATP-binding component (CR033) but no substrate-binding component (Table 8.2). The genome completely lacks genes for the flagellar apparatus, which might serve as an alternative to transporters in some other symbiotic bacteria (Shigenobu et al., 2000).

The extreme AT-richness in the genome may result from the loss of DNA repair functions in *Carsonella*. The genome of *Carsonella*-Pv lacks uracil-DNA glycosylase (*ung*), which removes uracil residues from DNA, and dUTPase (*dut*), which prevents dUTP from being misincorporated into DNA. The *dut* gene is conserved in all other sequenced endosymbionts, and the *ung* gene is retained in them except *Buchnera*-Sg. The lack of these two enzymes is the only substantial difference in repair gene sets between *Carsonella* and other bacteriocyte endosymbionts and might underlie the extreme AT-richness of the *Carsonella* genome. The genome also lacks other genes for DNA repair, such as *polA*, *nth*, *mutT*, *mutS*, *mutY*, and *mutL*. Moreover, *Carsonella*-Pv has lost most other genes involved in DNA metabolism. With respect to DNA replication, the genome retains *recA* (CR110), *dnaB* (CR055), *dnaG* (CR058), and genes encoding alpha (*dnaE*, CR023) and epsilon (*dnaQ*, CR087) subunits of DNA polymerase III, but lacks *dnaA*, *dnaN*, *dnaX*, *priA*, *recBCD*, *sbcB*, *gyrAB*, *lig*, and *topA* (Table 8.2). As for transcription, genes for the alpha (*rpoA*, CR135), beta (*rpoB*, CR162) and beta' (*rpoC*, CR161) subunits of the core RNA polymerase are retained. *Carsonella*-Pv has only one sigma factor (*rpoD*, CR059), whereas other sequenced bacteriocyte symbionts retain two sigma factors (*rpoD* and *rpoH*). Genes for transcription elongation and termination, such as *nusA*, *nusB*, *nusG*, *greA*, *deaD*, and *rho* are missing. The genome retains 15 genes for aminoacyl-tRNA synthetase, but lacks *argS*, *asnS*, *cysS*, *glyS*, *pheT*, *pros*, and *thrS*.

Transcriptomics of the aphid bacteriocyte

Seeking the host's role

The complete genome sequences of four lineages of *Buchnera* gave comprehensive information on potential functions of *Buchnera* in the symbiotic system (Shigenobu et al., 2000; Tamas et al., 2002; van Ham et al., 2003; Pérez-Brocal et al., 2006). The genomes were 0.42–0.65 Mb in size, which encoded 360–580 proteins (Table 8.1). The gene composition corroborated the experimental data that primary role of *Buchnera* in the bacteriocyte symbiosis is to provide their hosts with essential amino acids (Baumann et al., 1995; Sasaki and Ishikawa, 1995; Febvay et al., 1995; Nakabachi and Ishikawa, 1997; Douglas, 1998). Gene inven-

tories of *Buchnera*-APS, *Buchnera*-Sg, and *Buchnera*-Bp indicated that they can synthesize riboflavin (vitamin B₂) that are also scarce in the phloem sap (Ziegler, 1975), supporting the physiological data (Nakabachi and Ishikawa, 1999).

Although the genome studies of *Buchnera* clearly demonstrated its mutualistic nature, providing some insights into how the symbiotic system operates, they also raised a number of questions that can be answered only by understanding the host's function. Although the streamlining of the *Buchnera* genomes is not so extreme as the case of *Carsonella*, many genes that seem to be essential for their own living are missing from the genomes. For example, *Buchnera* lack most genes for biosyntheses of nonessential amino acids (amino acids that metazoa can synthesize: alanine, asparagine, aspartic acid, cysteine, glutamic acid, glutamine, glycine, proline, serine, and tyrosine), and phospholipids, suggesting that they can synthesize neither nonessential amino acids nor their own cell membrane. Most genes for transcriptional regulators are also lost, implying that *Buchnera* is hardly able to regulate their own metabolic and cellular activities, which has been supported by microarray data (Wilcox et al., 2003; Moran et al., 2005a). Such incomplete aspects of *Buchnera* functions should be compensated for by the activities of the host, but very little was known about function of the host bacteriocyte. To elucidate the host's role in the aphid-*Buchnera* system, the mRNA population of the host bacteriocyte of the pea aphid, *A. pisum*, was assessed by expressed sequence tag (EST) analysis and real-time quantitative RT-PCR (Nakabachi et al., 2005).

Selective upregulation of genes

Full-length cDNA library was constructed by using bacteriocytes that were isolated from a strain ISO, which is free from secondary symbionts. In total, 2,870 cDNA clones were sequenced from the 5' end. After removal of low-quality sequences and contaminants, 2,344 high-quality sequences were assembled into 336 nonredundant sequences (246 contigs + 90 singlets) (Table 8.3). BLASTX similarity searches followed by classification based on gene ontology (GO; The Gene Ontology Consortium, 2000) revealed that the bacteriocyte transcriptome contained significantly higher percentage of ESTs in the categories "amino acid metabolism," "defense response," and "transport" in comparison to two other whole body libraries (Hunter, et al., 2003; Sabater-Muñoz et al., 2005).

Amino acid metabolisms complementary to Buchnera

Real-time quantitative RT-PCR further verified the conspicuous upregulation of genes related to amino acid metabolisms (Figure 8.2A). The bacteriocyte is the cell for harboring *Buchnera*, whose primary role is the synthesis of essential amino acids (Sasaki and Ishikawa, 1995; Febvay et al., 1995; Douglas, 1998). The genome analyses of *Buchnera* showed that these symbionts are specialized in production of essential amino acids: genes for synthesis of essential amino acids are retained, whereas genes for synthesis of nonessential amino acids are mostly lost (Shigenobu et al., 2000; Tamas et al., 2002; van Ham et al., 2003; Pérez-Brocal et al., 2006). In the bacteriocyte, therefore, it is expected that essential amino acids are supplied by *Buchnera*, whereas nonessential amino acids must be synthesized in excess by the host. In agreement with the expectation, genes most highly expressed in the bacteriocyte include those for utilization of essential amino acids (those for cationic amino acid transporter 2, Henna [an enzyme with activities of phenylalanine 4-monooxygenase and tryptophan 5-monooxygenase, which are involved in catabolisms of L-phenylalanine and L-tryptophan, respectively], and glutaryl-CoA dehydrogenase [an enzyme that cata-

Table 8.3 Transcripts Detected in the Aphid Bacteriocyte

Local ID	Number of ESTs	Protein Homologue	Source Organism	Accession Number	E-value
R2C00037	134	Lysozyme i-1	<i>Anopheles gambiae</i>	AAT51799	2.3E-35
R2C00204	71	Lysozyme i-1	<i>Anopheles gambiae</i>	AAT51799	1.1E-34
R2C00172	70	ADP / ATP translocase	<i>Drosophila melanogaster</i>	AAB31734	6.9E-103
R2C00040	55	Glutamine synthetase 2 (CG1743; Gs2)	<i>Drosophila melanogaster</i>	NP_727525	2.2E-80
R2C00101	55	alpha-Tubulin at 84B (CG1913; alphaTub84B)	<i>Drosophila melanogaster</i>	NP_476772	9.3E-116
R2C00059	45	no hits			
R2C00253	34	Cytosolic malate dehydrogenase	<i>Homo sapiens</i>	NP_005908	1.8E-66
R2C00050	33	Ribosomal protein S9 (CG3395; RpS9)	<i>Drosophila melanogaster</i>	NP_524004	1.8E-95
R2C00038	27	Cationic amino acid transporter 2	<i>Homo sapiens</i>	NP_003037	1.9E-46
R2C00023	26	Heat shock protein cognate 4 (CG4264; Hsc70-4)	<i>Drosophila melanogaster</i>	NP_524356	1.1E-92
R2C00089	26	Putative inorganic phosphate cotransporter (CG8098; Picot)	<i>Drosophila melanogaster</i>	Q9V7S5	4.5E-28
R2C00113	26	Ribosomal protein L15 (CG17420; RpL15)	<i>Drosophila melanogaster</i>	NP_652103	1.1E-88
R2C00020	25	Probable mitochondrial oxaloacetate transport protein	<i>Schizosaccharomyces pombe</i>	NP_593169	1.7E-22
R2C00100	24	Phosphoenolpyruvate carboxykinase (CG17725; Pepck)	<i>Drosophila melanogaster</i>	NP_523784	2.2E-73
R2C00132	24	Glycine cleavage system T protein	<i>Homo sapiens</i>	NP_000472	1.4E-41
R2C00244	23	ATP synthase subunit c	<i>Manduca sexta</i>	Q9U505	1.0E-46
R2C00124	22	Ribosomal protein S14a (CG1524; RpS14a)	<i>Drosophila melanogaster</i>	NP_524884	2.5E-76
R2C00011	21	no hits			

Continued.

Table 8.3 Transcripts Detected in the Aphid Bacteriocyte (Continued)

Local ID	Number of ESTs	Protein Homologue	Source Organism	Accession Number	E-value
R2C00108	21	Ribosomal protein S3A (CG2168; RpS3A)	<i>Drosophila melanogaster</i>	NP_524618	1.6E-83
R2C00022	20	Diacetyl/L-xylulose reductase	<i>Xenopus tropicalis</i>	NP_989100	1.6E-30
R2C00106	20	5'-Nucleotidase precursor	<i>Lutzomyia longipalpis</i>	Q9XZ43	2.2E-47
R2C00013	18	no hits			
R2C00042	18	no hits			
R2C00130	18	Calmodulin (CG8472; Cam)	<i>Drosophila melanogaster</i>	NP_523710	6.2E-81
R2C00041	17	Ribosomal protein L19 (CG2746; RpL19)	<i>Drosophila melanogaster</i>	NP_476631	1.1E-90
R2C00055	17	Inwardly rectifying potassium channel 2 (CG4370; Irk2)	<i>Drosophila melanogaster</i>	NP_651149	1.5E-59
R2C00131	17	Protein translation factor SUI1 homologue (CG17737)	<i>Drosophila melanogaster</i>	Q9VZS3	9.8E-54
R2C00014	16	CG13849-PA	<i>Drosophila melanogaster</i>	NP_651040	1.5E-29
R2C00097	16	no hits			
R2C00114	16	Ribosomal protein S3 (CG6779; RpS3)	<i>Drosophila melanogaster</i>	NP_476632	7.4E-85
R2C00115	16	Cell cycle regulator p21 protein, Wos2p	<i>Schizosaccharomyces pombe</i>	NP_594586	2.6E-23
R2C00187	16	no hits			
R2C00195	16	CG17221-PA	<i>Drosophila melanogaster</i>	NP_608746	5.1E-19
R2C00061	15	Ribosomal protein L8 (CG1263; RpL8)	<i>Drosophila melanogaster</i>	NP_524726	1.2E-111
R2C00063	15	CG3975-PA	<i>Drosophila melanogaster</i>	NP_609743	4.3E-12
R2C00067	15	no hits			
R2C00092	15	Ribosomal protein L31 (CG1821; RpL31)	<i>Drosophila melanogaster</i>	NP_610503	1.9E-45
R2C00094	15	no hits			

R2C00007	14	no hits					
R2C00027	14	no hits					
R2C00034	14	CG5567-PA		<i>Drosophila melanogaster</i>	NP_649015	2.4E-43	
R2C00076	14	no hits					
R2C00084	14	Carbonyl reductase (NADPH) 1		<i>Homo sapiens</i>	NP_001748	7.7E-39	
R2C00087	14	no hits					
R2C00232	14	no hits					
R2C00004	13	Ribosomal protein L7 (CG4897; RpL7)		<i>Drosophila melanogaster</i>	NP_523531	7.4E-67	
R2C00031	13	Twinstar (CG4254; tsr)		<i>Drosophila melanogaster</i>	NP_477034	8.5E-67	
R2C00074	13	Ribosomal protein L26 (CG6846;RpL26)		<i>Drosophila melanogaster</i>	NP_649070	1.7E-58	
R2C00077	13	Receptor of activated protein kinase C 1 (CG7111; Rack1)		<i>Drosophila melanogaster</i>	NP_477269	6.9E-92	
R2C00081	13	no hits					
R2C00107	13	Henna (CG7399, Hn)		<i>Drosophila melanogaster</i>	NP_523963	7.0E-54	
R2C00128	13	CG6767-PB		<i>Drosophila melanogaster</i>	NP_729528	1.2E-105	
R2C00161	13	Vacuolar proton pump, 21-kD subunit		<i>Homo sapiens</i>	NP_004038	2.8E-49	
R2C00016	12	no hits					
R2C00029	12	CG13907-PA		<i>Drosophila melanogaster</i>	NP_612069	1.8E-55	
R2C00116	12	CG10877-PA		<i>Drosophila melanogaster</i>	NP_650894	2.4E-38	
R2C00123	12	no hits					
R2C00234	12	no hits					
R2C00135	11	Lamina ancestor (CG10645; lama)		<i>Drosophila melanogaster</i>	NP_729059	1.8E-20	
R2C00159	11	CG8983-PA		<i>Drosophila melanogaster</i>	NP_725084	1.0E-47	
R2C00173	11	no hits					
R2C00188	11	CG6180-PA		<i>Drosophila melanogaster</i>	NP_609588	3.9E-64	
R2C00001	10	Vacuolar proton pump d subunit 1 (CG2934; VhaAC39)		<i>Drosophila melanogaster</i>	NP_570080	5.6E-58	

Continued.

Table 8.3 Transcripts Detected in the Aphid Bacteriocyte (Continued)

Local ID	Number of ESTs	Protein Homologue	Source Organism	Accession Number	E-value
R2C00036	10	CG2185-PA	<i>Drosophila melanogaster</i>	NP_649568	3.1E-61
R2C00039	10	no hits			
R2C00068	10	Rab40c, member RAS oncogene family	<i>Mus musculus</i>	NP_631893	7.5E-33
R2C00193	10	Rare lipoprotein A precursor	<i>Yersinia pestis</i>	Q8ZDG6	7.3E-11
R2C00006	9	String of pearls (CG5920; sop)	<i>Drosophila melanogaster</i>	NP_476874	1.4E-98
R2C00009	9	Ribosomal protein L26 (CG6846; RpL26)	<i>Drosophila melanogaster</i>	NP_649070	3.1E-59
R2C00028	9	CG1101-PA	<i>Drosophila melanogaster</i>	NP_651968	1.8E-44
R2C00066	9	Glutathione S transferase D1 (CG10045; GstD1)	<i>Drosophila melanogaster</i>	NP_524326	1.9E-63
R2C00088	9	Hypothetical protein MG08000.4	<i>Magnaporthe grisea</i> 70-15	EAA57031	8.9E-15
R2C00099	9	CG12781-PA, PB (nahoda)	<i>Drosophila melanogaster</i>	NP_523815, NP_726264	9.0E-13
R2C00139	9	Ribosomal protein L36A (CG7424; RpL36A)	<i>Drosophila melanogaster</i>	NP_609179	1.2E-54
R2C00151	9	Ribosomal protein L9 (CG6141; RpL9)	<i>Drosophila melanogaster</i>	NP_477161	3.8E-80
R2C00216	9	CG2254-PA	<i>Drosophila melanogaster</i>	NP_572436	1.0E-25
R2C00012	8	Dihydroliipoamide S-acetyltransferase (53.5 kD)	<i>Caenorhabditis elegans</i>	NP_506579	3.7E-40
R2C00017	8	no hits			
R2C00025	8	ATP synthase alpha chain, mitochondrial precursor (Bellwether) (CG3612; blw)	<i>Drosophila melanogaster</i>	NP_726243	3.8E-88
R2C00095	8	Adenine phosphoribosyltransferase (CG18315; Aprt)	<i>Drosophila melanogaster</i>	NP_476637	8.8E-31
R2C00102	8	Ribosomal protein S15A (CG2033; RpS15Aa)	<i>Drosophila melanogaster</i>	NP_727690	3.2E-66

R2C00104	8	Ribosomal protein S26 (CG10305; RpS26)	<i>Drosophila melanogaster</i>	NP_724109	7.5E-50
R2C00105	8	CG7332-PA	<i>Drosophila melanogaster</i>	NP_573338	9.4E-21
R2C00110	8	CG8947-PA	<i>Drosophila melanogaster</i>	NP_620470	4.5E-38
R2C00112	8	Bax inhibitor-1	<i>Mus musculus</i>	NP_080945	1.3E-24
R2C00143	8	Hypothetical protein MGC4614	<i>Homo sapiens</i>	NP_077270	6.6E-36
R2C00144	8	no hits			
R2C00158	8	Ribosomal protein L11 (CG7726; RpL11)	<i>Drosophila melanogaster</i>	NP_477054	8.7E-86
R2C00162	8	Dihydropteridine reductase (CG4665; Dhpr)	<i>Drosophila melanogaster</i>	NP_523990	3.1E-45
R2C00166	8	CG32549-PD	<i>Drosophila melanogaster</i>	NP_573289	4.2E-59
R2C00186	8	Phosphogluconate dehydrogenase (CG3724; Pgd)	<i>Drosophila melanogaster</i>	NP_476860	4.0E-83
R2C00208	8	Elongation factor 1alpha100E (CG1873; Eflalpha100E)	<i>Drosophila melanogaster</i>	NP_524611	1.6E-101
R2C00248	8	no hits			
R2C00008	7	Heat shock protein 68 (CG5436; Hsp68)	<i>Drosophila melanogaster</i>	NP_524474	2.0E-63
R2C00019	7	Ribosomal protein P2 (CG4918; RpLP2)	<i>Drosophila melanogaster</i>	NP_523764	8.8E-30
R2C00030	7	Vacuolar proton pump B subunit (CG17369; Vha55)	<i>Drosophila melanogaster</i>	NP_731726	2.0E-98
R2C00073	7	no hits			
R2C00079	7	Vacuolar proton pump d subunit 1 (CG2934; VhaAC39)	<i>Drosophila melanogaster</i>	NP_570080	1.3E-92
R2C00086	7	Mitochondrial glutamate carrier 1	<i>Homo sapiens</i>	NP_078974	1.6E-41
R2C00157	7	Senescence marker protein-30 (CG7390; smp-30)	<i>Drosophila melanogaster</i>	NP_524353	1.0E-19
R2C00217	7	Ferrochelatase (CG2098; ferrochelatase)	<i>Drosophila melanogaster</i>	NP_524613	8.7E-63
R2C00224	7	Malate dehydrogenase (CG5889; Mdh)	<i>Drosophila melanogaster</i>	CAB64263	8.6E-82

Continued.

Table 8.3 Transcripts Detected in the Aphid Bacteriocyte (Continued)

Local ID	Number of ESTs	Protein Homologue	Source Organism	Accession Number	E-value
R2C00241	7	Ornithine aminotransferase (CG8782; Oat)	<i>Drosophila melanogaster</i>	Q9VW26	8.6E-51
R2C00021	6	CG17521-PA, PB (Qm)	<i>Drosophila melanogaster</i>	NP_651954, NP_730773	1.9E-113
R2C00043	6	26S protease regulatory subunit 8 (CG1489; Pros45)	<i>Drosophila melanogaster</i>	O18413	3.3E-85
R2C00046	6	Maroon-like (CG1692; mal)	<i>Drosophila melanogaster</i>	NP_523423	4.3E-32
R2C00047	6	Histone H3.3A (CG5825; His3.3A), H3.3B (CG8989; His3.3B)	<i>Drosophila melanogaster</i>	NP_523479, NP_511095	2.4E-71
R2C00060	6	CG12400-PA	<i>Drosophila melanogaster</i>	NP_608750	8.1E-18
R2C00064	6	no hits			
R2C00080	6	Vacuolar proton pump F subunit (CG8210; Vha14)	<i>Drosophila melanogaster</i>	NP_476969	1.2E-57
R2C00082	6	Thioredoxin peroxidase 1 (CG1633; Jafracl)	<i>Drosophila melanogaster</i>	NP_477510	8.0E-74
R2C00085	6	CG11594-PA, PC	<i>Drosophila melanogaster</i>	NP_728918, NP_728919	5.7E-55
R2C00090	6	Glutaryl-coenzyme A dehydrogenase	<i>Homo sapiens</i>	NP_000150	3.2E-66
R2C00091	6	CG15890-PA	<i>Drosophila melanogaster</i>	NP_572968	1.2E-25
R2C00103	6	3-Phosphoglycerate dehydrogenase	<i>Mus musculus</i>	NP_058662	1.8E-52
R2C00111	6	Retinol dehydrogenase 12 (all-trans and 9-cis)	<i>Homo sapiens</i>	NP_689656	1.7E-44
R2C00119	6	Eukaryotic initiation factor 4a (CG9075; eIF-4a)	<i>Drosophila melanogaster</i>	NP_723137	1.9E-67
R2C00122	6	Vacuolar protein sorting 18	<i>Homo sapiens</i>	NP_065908	1.4E-54
R2C00137	6	Cut up (CG6998; ctp)	<i>Drosophila melanogaster</i>	NP_525075	7.8E-48

R2C00141	6	CG17266-PA	<i>Drosophila melanogaster</i>	NP_610224	2.3E-75
R2C00142	6	Ribosomal protein S15 (CG8332; RpS15)	<i>Drosophila melanogaster</i>	NP_611136	1.5E-63
R2C00147	6	LRP16 protein	<i>Homo sapiens</i>	NP_054786	3.9E-44
R2C00171	6	no hits			
R2C00192	6	Abnormal wing discs (CG2210; awd)	<i>Drosophila melanogaster</i>	NP_476761	1.2E-53
R2C00196	6	Cystathionine gamma-lyase (43.1 kD) (2H346)	<i>Caenorhabditis elegans</i>	NP_495449	9.9E-40
R2C00211	6	no hits			
R2C00229	6	Elongation factor 1 beta (CG6341; Ef1beta)	<i>Drosophila melanogaster</i>	NP_524808	1.2E-51
R2C00233	6	CG15261-PA	<i>Drosophila melanogaster</i>	NP_609747	8.0E-27
R2C00003	5	Ribosomal protein S5 (CG7014; RpS5b)	<i>Drosophila melanogaster</i>	NP_650407	4.2E-77
R2C00005	5	Ribosomal protein L18 (CG8615; RpL18)	<i>Drosophila melanogaster</i>	NP_648091	7.1E-72
R2C00026	5	Actin 42A (CG12051; Act42A), 5C (CG4027-PB; Act5C)	<i>Drosophila melanogaster</i>	NP_523625, NP_727048	1.4E-74
R2C00044	5	no hits			
R2C00045	5	Secretory carrier membrane protein 1 isoform 1, isoform 2	<i>Homo sapiens</i>	NP_004857, NP_438173	2.1E-15
R2C00049	5	CG4090-PA	<i>Drosophila melanogaster</i>	NP_650611	8.6E-13
R2C00058	5	Vitellogenic carboxypeptidase precursor	<i>Aedes aegypti</i>	P42660	4.3E-26
R2C00078	5	Elongation factor 2b (CG2238; Ef2b)	<i>Drosophila melanogaster</i>	NP_525105	1.6E-98
R2C00083	5	no hits			
R2C00096	5	Ribosomal protein L17A (CG3661; RpL23)	<i>Drosophila melanogaster</i>	NP_523813	1.7E-70
R2C00121	5	no hits			
R2C00126	5	Rhodnius prolixus MIP-like protein	<i>Rhodnius prolixus</i>	CAC13959	2.7E-11

Continued.

Table 8.3 Transcripts Detected in the Aphid Bacteriocyte (Continued)

Local ID	Number of ESTs	Protein Homologue	Source Organism	Accession Number	E-value
R2C00127	5	Phosphoribosyl pyrophosphate synthetase 1	<i>Homo sapiens</i>	NP_002755	2.0E-97
R2C00156	5	no hits			
R2C00167	5	CG7787-PA	<i>Drosophila melanogaster</i>	NP_609209	1.5E-21
R2C00169	5	CG40049-PA, PB	<i>Drosophila melanogaster</i>	EAA46075, EAA46076	1.1E-28
R2C00180	5	no hits			
R2C00190	5	Cytochrome c oxidase subunit Va	<i>Rhyzopertha dominica</i>	AAL17607	1.4E-42
R2C00201	5	Inositol(myo)-1(or 4)-monophosphatase 1	<i>Homo sapiens</i>	NP_005527	5.3E-29
R2C00203	5	Splicing factor SRp54 (CG4602; Srp54)	<i>Drosophila melanogaster</i>	NP_477347	9.2E-49
R2C00227	5	Succinyl coenzyme A synthetase flavoprotein subunit (CG17246; Scs-fp)	<i>Drosophila melanogaster</i>	NP_477210	2.2E-76
R2C00251	5	CG11951-PA	<i>Drosophila melanogaster</i>	NP_651688	1.5E-35
R2C00010	4	CG7458-PA	<i>Drosophila melanogaster</i>	NP_649374	1.6E-27
R2C00018	4	Ribosomal protein S12 (CG11271; RpS12)	<i>Drosophila melanogaster</i>	NP_729865	1.4E-31
R2C00032	4	CG17323-PA	<i>Drosophila melanogaster</i>	NP_609910	4.6E-18
R2C00033	4	CG9165-PA	<i>Drosophila melanogaster</i>	NP_612103	9.1E-47
R2C00053	4	no hits			
R2C00069	4	no hits			
R2C00072	4	Mitochondrial ribosomal protein L20 (CG11258; mRpL20)	<i>Drosophila melanogaster</i>	NP_524051	8.1E-41
R2C00075	4	CG5569-PA	<i>Drosophila melanogaster</i>	NP_611840	2.9E-18

R2C00093	4	Phosphoserine aminotransferase isoform 1	<i>Homo sapiens</i>	NP_478059	4.4E-34
R2C00133	4	Carbonyl reductase (NADPH) 1	<i>Homo sapiens</i>	NP_001748	2.2E-35
R2C00154	4	Ribosomal protein L46 (CG3997-PA; RpL39)	<i>Drosophila melanogaster</i>	NP_477314	9.6E-21
R2C00164	4	no hits			
R2C00168	4	Cytochrome c oxidase polypeptide IV	<i>Drosophila simulans</i>	AAP88302	1.6E-32
R2C00170	4	no hits			
R2C00175	4	no hits			
R2C00189	4	Adenine phosphoribosyltransferase (20.2 kD) (1G247)	<i>Caenorhabditis elegans</i>	NP_491663	5.0E-29
R2C00197	4	Solute carrier family 25 (mitochondrial carrier; oxoglutarate carrier), member 11	<i>Homo sapiens</i>	NP_003553	1.4E-41
R2C00198	4	Choline phosphotransferase 1	<i>Homo sapiens</i>	NP_064629	1.3E-25
R2C00200	4	CG10711-PA	<i>Drosophila melanogaster</i>	NP_648660	1.2E-52
R2C00209	4	no hits			
R2C00213	4	CG5941-PA	<i>Drosophila melanogaster</i>	NP_572288	5.5E-67
R2C00214	4	Conserved hypothetical protein	<i>Wolbachia pipientis</i> wMel	NP_966741	7.1E-22
R2C00215	4	no hits			
R2C00218	4	Vacuolar proton pump G subunit (CG6213; VhaI3)	<i>Drosophila melanogaster</i>	NP_477437	1.1E-40
R2C00228	4	CG10171-PA	<i>Drosophila melanogaster</i>	NP_648639	4.9E-40
R2C00246	4	CG8093-PA	<i>Drosophila melanogaster</i>	NP_611020	2.5E-20
R2C00035	3	Bax inhibitor-1	<i>Mus musculus</i>	NP_080945	1.2E-24
R2C00048	3	no hits			
R2C00054	3	Sanpodo (CG1539; spdo)	<i>Drosophila melanogaster</i>	NP_733385	4.1E-77
R2C00056	3	no hits			
R2C00065	3	ENSANGP000000015016	<i>Anopheles gambiae</i>	XP_308743	2.7E-14

Continued.

Table 8.3 Transcripts Detected in the Aphid Bacteriocyte (Continued)

Local ID	Number of ESTs	Protein Homologue	Source Organism	Accession Number	E-value
R2C00070	3	Small nuclear ribonucleoprotein at 69D (CG10753-PA; snRNP69D)	<i>Drosophila melanogaster</i>	NP_524774	9.4E-48
R2C00098	3	Dihydrofolate reductase (CG14887; Dhfr)	<i>Drosophila melanogaster</i>	NP_732147	9.7E-38
R2C00129	3	no hits			
R2C00140	3	Proteasome beta 4 subunit	<i>Homo sapiens</i>	NP_002787	1.9E-38
R2C00148	3	no hits			
R2C00149	3	Antennal protein 10 (CG6642; a10)	<i>Drosophila melanogaster</i>	NP_524121	6.0E-14
R2C00153	3	no hits			
R2C00165	3	Ferritin 1 heavy chain homologue (CG2216; Fer1HCH)	<i>Drosophila melanogaster</i>	NP_524873	1.7E-37
R2C00176	3	no hits			
R2C00177	3	no hits			
R2C00178	3	unknown	Environmental sequence	EAE11505	1.8E-51
R2C00179	3	no hits			
R2C00181	3	no hits			
R2C00182	3	no hits			
R2C00183	3	Acetyl-CoA transporter (2J539)	<i>Caenorhabditis elegans</i>	NP_495969	2.4E-40
R2C00184	3	no hits			
R2C00191	3	CG7207-PA	<i>Drosophila melanogaster</i>	NP_648199	6.1E-38
R2C00205	3	Ribosomal protein S19 (CG4464; RpS19a)	<i>Drosophila melanogaster</i>	NP_523376	2.4E-47
R2C00223	3	CG8680-PA	<i>Drosophila melanogaster</i>	NP_608909	3.7E-35
R2C00225	3	no hits			
R2C00226	3	no hits			
R2C00230	3	6-Pyruvoyl-tetrahydropterin synthase	<i>Mus musculus</i>	NP_035350	1.8E-47

R2C00231	3	Similar to putative growth hormone like protein-1	<i>Mus musculus</i>	XP_354884	5.8E-12
R2C00239	3	Fatty acid desaturase	<i>Drosophila melanogaster</i>	CAB52474	6.3E-53
R2C00015	2	no hits			
R2C00051	2	CG17870-PA, PB, PC, PG	<i>Drosophila melanogaster</i>	NP_724885, NP_724886, NP_724887, NP_724889	1.7E-49
R2C00052	2	Malate dehydrogenase (CG5889; Mdh)	<i>Drosophila melanogaster</i>	CAB64263	1.2E-48
R2C00057	2	CG10823-PA, PB	<i>Drosophila melanogaster</i>	NP_65096, NP_732614	4.4E-13
R2C00062	2	CG4692-PA, PB	<i>Drosophila melanogaster</i>	NP_726463, NP_611940	5.6E-43
R2C00109	2	no hits			
R2C00117	2	F-box and leucine-rich repeat protein 2	<i>Mus musculus</i>	NP_848739	1.3E-28
R2C00120	2	CG32230-PA, PB	<i>Drosophila melanogaster</i>	NP_730777, NP_730778	1.2E-18
R2C00134	2	CG4798-PB, PC, PD	<i>Drosophila melanogaster</i>	NP_725672, NP_725673, NP_725674	2.3E-18
R2C00136	2	CYcloPhilin (21.9 kD) (cyp-5)	<i>Caenorhabditis elegans</i>	NP_493624	4.5E-60
R2C00138	2	unknown	Environmental sequence	EAE11505	6.4E-22
R2C00145	2	Ornithine decarboxylase antizyme	<i>Homo sapiens</i>	AA82155	8.3E-17
R2C00146	2	no hits			
R2C00150	2	CG2091-PA	<i>Drosophila melanogaster</i>	NP_649582	3.3E-14
R2C00152	2	Cathepsin L	<i>Aphis gossypii</i>	CAD33266	7.6E-82
R2C00155	2	Heat shock protein 68 (CG5436; Hsp68)	<i>Drosophila melanogaster</i>	NP_524474	2.3E-66
R2C00160	2	Cytochrome c oxidase polypeptide VIb	<i>Drosophila simulans</i>	AAP88308	6.7E-22
R2C00163	2	Enoyl coenzyme A hydratase domain containing 3	<i>Homo sapiens</i>	NP_078969	1.7E-17

Continued.

Table 8.3 Transcripts Detected in the Aphid Bacteriocyte (Continued)

Local ID	Number of ESTs	Protein Homologue	Source Organism	Accession Number	E-value
R2C00174	2	Ribosomal protein L37-A (CG9091-PA; RpL37a)	<i>Drosophila melanogaster</i>	NP_573005	3.9E-44
R2C00185	2	Motile sperm domain containing 1	<i>Homo sapiens</i>	NP_062456	2.0E-28
R2C00194	2	CG5010-PA	<i>Drosophila melanogaster</i>	NP_573196	1.0E-35
R2C00202	2	Pyridoxine 5'-phosphate oxidase	<i>Homo sapiens</i>	NP_060599	4.9E-26
R2C00206	2	CG3321-PA, PB	<i>Drosophila melanogaster</i>	NP_650356, NP_731903	4.1E-16
R2C00210	2	Senescence marker protein-30 (CG7390; smp-30)	<i>Drosophila melanogaster</i>	NP_524353	9.2E-21
R2C00212	2	Actin 5C (CG4027; Act5C)	<i>Drosophila melanogaster</i>	NP_727048	3.5E-127
R2C00219	2	no hits			
R2C00220	2	CG11015-PA	<i>Drosophila melanogaster</i>	NP_609046	2.3E-34
R2C00221	2	no hits			
R2C00222	2	no hits			
R2C00235	2	{beta}1,4-N-acetyl-galactosaminyltransferase (43.9 kD) (1C94)	<i>Caenorhabditis elegans</i>	NP_490872	4.2E-27
R2C00236	2	Succinate dehydrogenase complex, subunit C, integral membrane protein	<i>Mus musculus</i>	NP_079597	4.8E-18
R2C00237	2	CG5033-PA	<i>Drosophila melanogaster</i>	NP_611270	2.3E-51
R2C00238	2	Adenine phosphoribosyltransferase (CG18315; Aprt)	<i>Drosophila melanogaster</i>	NP_476637	9.1E-31
R2C00240	2	Glyceraldehyde 3 phosphate dehydrogenase 1 (CG12055; Gapdh1)	<i>Drosophila melanogaster</i>	NP_525108	4.6E-70
R2C00242	2	Mitochondrial import inner membrane translocase subunit Tim9A (CG1660; Tim9a)	<i>Drosophila melanogaster</i>	NP_572881	1.9E-16

R2C00243	2	CG1236-PA	<i>Drosophila melanogaster</i>	NP_649579	6.8E-37
R2C00247	2	no hits			
R2C00249	2	no hits			
R2C00250	2	CG4090-PA	<i>Drosophila melanogaster</i>	NP_650611	1.7E-14
R2C00252	2	CG1709-PB, PD	<i>Drosophila melanogaster</i>	NP_733274, NP_733275	3.3E-88
R2C00254	2	CG13377-PA	<i>Drosophila melanogaster</i>	NP_569835	8.6E-11
BCA001015	1	no hits			
BCA001021	1	no hits			
BCA001028	1	CG12877-PA, PB, CG5516-PA	<i>Drosophila melanogaster</i>	NP_651584, NP_733234, NP_650515	1.6E-12
BCA002027	1	Gelsolin (CG1106; Gel)	<i>Drosophila melanogaster</i>	NP_524865	1.4E-17
BCA002044	1	CG4686-PA	<i>Drosophila melanogaster</i>	NP_650837	1.0E-12
BCA002060	1	no hits			
BCA003014	1	Ribosomal protein S13 (CG13389; RpS13)	<i>Drosophila melanogaster</i>	NP_476938	1.2E-67
BCA005032	1	ATPase coupling factor 6 (CG4412; ATPsyn-Cf6)	<i>Drosophila melanogaster</i>	NP_477194	2.5E-12
BCA005089	1	Phosphoglucose isomerase (CG8251; Pgi)	<i>Drosophila melanogaster</i>	NP_523663	2.6E-19
BCA006012	1	CG12107-PA	<i>Drosophila melanogaster</i>	NP_610314	1.5E-13
BCA007043	1	Ribosomal protein S9 (CG3395; RpS9)	<i>Drosophila melanogaster</i>	NP_524004	5.7E-81
BCA008009	1	Ribosomal protein L26 (CG6846; RpL26)	<i>Drosophila melanogaster</i>	NP_649070	2.7E-51
BCA008015	1	CG3309-PA	<i>Drosophila melanogaster</i>	NP_572216	6.4E-31
BCA008045	1	no hits			
BCA008048	1	CG3603-PA	<i>Drosophila melanogaster</i>	NP_570046	1.1E-38

Continued.

Table 8.3 Transcripts Detected in the Aphid Bacteriocyte (Continued)

Local ID	Number of ESTs	Protein Homologue	Source Organism	Accession Number	E-value
BCA009041	1	CG12393-PA, PB	<i>Drosophila melanogaster</i>	NP_608984, NP_723149	1.3E-40
BCA009044	1	no hits			
BCA009073	1	CG7580-PA	<i>Drosophila melanogaster</i>	NP_648985	2.0E-19
BCA010006	1	CG9140-PA	<i>Drosophila melanogaster</i>	NP_608987	3.6E-84
BCA010027	1	SPARC (secreted protein acidic and rich in cysteine)	<i>Artemia franciscana</i>	BAB20042	6.5E-29
BCA011030	1	no hits			
BCA011048	1	no hits			
BCA011076	1	NADH:ubiquinone reductase 75kD subunit precursor (CG2286; ND75)	<i>Drosophila melanogaster</i>	NP_511083	2.6E-77
BCA012002	1	no hits			
BCA012018	1	CG4630-PA	<i>Drosophila melanogaster</i>	NP_610847	8.2E-16
BCA012033	1	CG15890-PA	<i>Drosophila melanogaster</i>	NP_572968	9.1E-18
BCA012039	1	CG6084-PA	<i>Drosophila melanogaster</i>	NP_648484	1.4E-47
BCA013007	1	Fructose-1,6-bisphosphatase (CG31692-PA; fbp)	<i>Drosophila melanogaster</i>	NP_610001	5.5E-62
BCA013035	1	no hits			
BCA013048	1	no hits			
BCA013050	1	no hits			
BCA013064	1	Cytochrome oxidase subunit I	<i>Diuraphis noxia</i>	AAO23131	3.8E-88
BCA013079	1	ABC transporter expressed in trachea (CG2969; Atet)	<i>Drosophila melanogaster</i>	NP_523471	4.8E-28
BCA014016	1	CG6198-PA	<i>Drosophila melanogaster</i>	NP_651226	2.8E-56
BCA014030	1	Cationic amino acid transporter 1	<i>Homo sapiens</i>	NP_003036	8.8E-35
BCA016027	1	Beadex (CG6500; Bx)	<i>Drosophila melanogaster</i>	NP_728184	1.1E-63

BCA016032	1	Superoxide dismutase 2 (Mn) (CG8905; Sod2)	<i>Drosophila melanogaster</i>	NP_476925	2.0E-29
BCA016036	1	Ras-like GTP-binding protein Rho1 (CG8416; Rho1)	<i>Drosophila melanogaster</i>	NP_477098	6.7E-66
BCA016065	1	no hits			
BCA016066	1	no hits			
BCA016071	1	CG31301-PA	<i>Drosophila melanogaster</i>	NP_650449	8.5E-13
BCA018012	1	no hits			
BCA019013	1	no hits			
BCA021017	1	CG40049-PA, PB	<i>Drosophila melanogaster</i>	EAA46075, EAA46076	6.5E-27
BCA021025	1	Macrophage migration inhibitory factor; MIF	<i>Amblyomma americanum</i>	AAG28339	7.6E-24
BCA021029	1	no hits			
BCA021032	1	no hits			
BCA021042	1	no hits			
BCA021054	1	Guanosine monophosphate reductase 2	<i>Homo sapiens</i>	NP_057660	4.0E-50
BCA022012	1	CG2813-PA	<i>Drosophila melanogaster</i>	NP_608536	1.3E-34
BCA022015	1	Ribosomal protein L9 (CG6141; RpL9)	<i>Drosophila melanogaster</i>	NP_477161	5.0E-18
BCA022035	1	Abnormal wing discs (CG2210; awd)	<i>Drosophila melanogaster</i>	NP_476761	6.0E-55
BCA022042	1	CG9166-PA	<i>Drosophila melanogaster</i>	NP_612105	3.8E-17
BCA022050	1	unknown	Environmental sequence	EAD41397	7.7E-18
BCA023018	1	no hits			
BCA023022	1	CG6265-PA, PB	<i>Drosophila melanogaster</i>	NP_651527, NP_733186	7.9E-17
BCA023025	1	no hits			
BCA023053	1	Thioredoxin reductase-1 (CG2151; Trxr-1)	<i>Drosophila melanogaster</i>	NP_511082	1.0E-47

Continued.

Table 8.3 Transcripts Detected in the Aphid Bacteriocyte (Continued)

Local ID	Number of ESTs	Protein Homologue	Source Organism	Accession Number	E-value
BCA024021	1	Phosphoglucose isomerase	<i>Gryllus veletis</i>	AAG15513	3.6E-16
BCA024038	1	no hits			
BCA024053	1	CG33138-PA	<i>Drosophila melanogaster</i>	NP_788342	4.2E-17
BCA025002	1	Cytochrome c proximal (CG17903; Cyt-c-p)	<i>Drosophila melanogaster</i>	NP_477176	5.9E-45
BCA026021	1	CG16707-PA, PB, PC, PD	<i>Drosophila melanogaster</i>	NP_729535, NP_729536, NP_648349, NP_729534	2.2E-22
BCA027002	1	Proteasome 25kD subunit (CG5266; Pros25)	<i>Drosophila melanogaster</i>	NP_524328	2.8E-83
BCA027014	1	no hits			
BCA027054	1	no hits			
BCA028031	1	no hits			
BCA029003	1	no hits			
BCA029023	1	no hits			
BCA030014	1	no hits			
BCA030024	1	no hits			
BCA030085	1	no hits			
BCA031009	1	Phosphatase and tensin homologue	<i>Homo sapiens</i>	NP_000305	2.8E-43
BCA032027	1	no hits			
BCA032058	1	Soluble NSF attachment protein (CG6625; Snap)	<i>Drosophila melanogaster</i>	NP_524180	5.1E-36
BCA032072	1	no hits			
BCA035001	1	Ribosomal protein L38	<i>Spodoptera frugiperda</i>	AAK92173	3.2E-30

BCA035046	1	no hits				
BCA035050	1	CG3625-PB		<i>Drosophila melanogaster</i>	NP_608514	3.7E-11
BCA036005	1	Cytochrome c oxidase subunit Va		<i>Rhyzopertha dominica</i>	AAL17607	1.3E-42
BCA036012	1	Acetyl-CoA transporter (2J539)		<i>Caenorhabditis elegans</i>	NP_495969	1.4E-43
BCA037073	1	CG6767-PA		<i>Drosophila melanogaster</i>	NP_648345	5.1E-104
BCA037074	1	no hits				
BCA038003	1	Ribosomal protein S8		<i>Apis mellifera</i>	AAC28863	2.7E-46
BCA038026	1	no hits				
BCA038038	1	no hits				
BCA038047	1	no hits				
BCA038054	1	no hits				
BCA038058	1	CG7888-PA, PB, PC		<i>Drosophila melanogaster</i>	NP_729651, NP_648425, NP_729652	7.7E-41
BCA038063	1	beta-Tubulin at 60D (CG3401; betaTub60D)		<i>Drosophila melanogaster</i>	NP_523842	1.2E-90
BCA038067	1	CG3132-PA		<i>Drosophila melanogaster</i>	NP_650142	1.1E-44

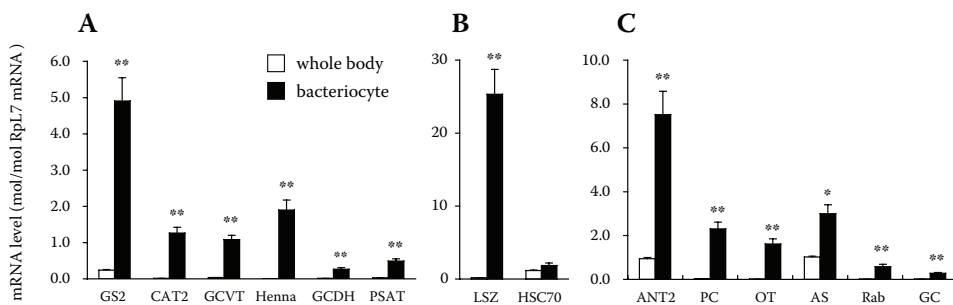


Figure 8.2 Quantitative RT-PCR of aphid genes expressed in the bacteriocyte. (A) Genes related to amino acid metabolism: GS2, glutamine synthetase 2; CAT2, cationic amino acid transporter 2; GCVT, glycine cleavage system T protein; Henna; GCDH, glutaryl-CoA dehydrogenase; PSAT, phosphoserine aminotransferase. (B) Genes related to defense response: LSZ, lysozyme; HSC70, heat shock protein cognate 4. Although the gene for HSC70 was among the most highly expressed transcripts in the category “defense response” (Table 8.3; R2C00023), quantitative RT-PCR revealed no significant upregulation of the gene in the bacteriocyte. (C) Genes related to transport: ANT2, ADP/ATP translocase; PC, inorganic phosphate cotransporter; OT, mitochondrial oxaloacetate transport protein; AS, ATP synthase subunit c; Rab; GC, mitochondrial glutamate carrier. Open columns, expression levels in the whole body; filled columns, expression levels in the bacteriocyte; bars, standard errors ($n = 10$). The expression levels are shown in terms of mRNA copies of target genes per copy of mRNA for ribosomal protein L7. Asterisks indicate statistically significant differences (Mann-Whitney U test; *, $p < 0.05$; **, $p < 0.01$). (Modified from Nakabachi, A., Shigenobu, S., Sakazume, N., Shiraki, T., Hayashizaki, Y., Carninci, P., Ishikawa, H., Kudo, T., and Fukatsu, T. [2005]. *Proc. Natl. Acad. Sci. U.S.A.* **102**: 5477–5482.)

lyzes the oxidative decarboxylation of glutaryl-CoA, which is involved in L-tryptophan metabolism and degradative pathways of L-lysine and L-hydroxylysine) and genes for synthesis of nonessential amino acids (those for glutamine synthetase 2 and phosphoserine aminotransferase [an enzyme that is involved in serine biosynthesis]). These results revealed an important aspect of the molecular basis of interdependency between the host and *Buchnera*.

Mysterious lysozyme homologues

The most abundant transcripts (205 clones in total, 8.7% of total ESTs in the bacteriocyte transcriptome) were classified into the GO category “defense response,” as they showed significant similarity to genes for lysozymes (Table 8.3), the enzymes that destroy bacterial cell walls (Bachali et al., 2002). Quantitative RT-PCR demonstrated that their expression level was 156 times higher in the bacteriocyte than in the whole body (Figure 8.2B). It is also notable that the level of the transcripts was strikingly higher, 25.3-fold, than that of the control gene for a ribosomal protein, verifying their extreme abundance in the aphid bacteriocyte.

Although their enzymatic activity is yet to be determined, it is of great interest as to why the bacteriocyte highly expresses genes characterized as having antibacterial functions. As electron microscopic studies reported putative lysosomal breakdown of *Buchnera* and secondary symbionts in the bacteriocyte (Hinde, 1971; Griffiths and Beck, 1973), the lysozyme homologues in the aphid bacteriocyte may function for the following purposes:

1. Removal of dead or senescent *Buchnera* cells: In the bacteriocyte, *Buchnera* cells are proliferating and dying at a certain rate. The lysozymes may be used to scavenge dead or senescent *Buchnera* cells to keep the symbiotic system in good conditions.
2. Control of *Buchnera* population: In general, the proliferation rate of prokaryotic cells is higher than that of eukaryotic cells. Thus, it is expected that the symbiont cells are potentially able to proliferate faster than the host cells. Because the overgrowth of the symbiont would lead to disruption of the symbiotic system, some control mechanism is required. Considering the absence of genes for regulating their own proliferation (e.g., genes for the quorum sensing system) in the *Buchnera* genome (Shigenobu et al., 2000), it appears plausible that the host aphid controls the *Buchnera* population by using, for example, lysozymes, although other mechanisms of control are also plausible.
3. Harvest of *Buchnera* cells for resource allocation: Previous studies reported that the number and the size of bacteriocytes remarkably decrease under certain physiological conditions that could impose nutritional stress (e.g., wing formation, starvation) (Hongoh and Ishikawa, 1994). These observations suggest that the *Buchnera*-harboring bacteriocytes might be potentially utilized as nutritional reservoir for the host aphid. The process of resource allocation might involve the lysozymes.
4. Elimination of microbial intruders: The cytoplasm of the bacteriocyte harbors a dense monoculture of *Buchnera*, suggesting that the cellular environment might potentially be suitable for bacterial proliferation. It is conceivable that the lysozymes attack foreign bacterial intruders to maintain the pure *Buchnera* population in the bacteriocyte. The observation that various non-*Buchnera* microorganisms proliferated in *Buchnera*-eliminated pea aphids in which bacteriocytes were atrophied (Nakabachi et al., 2003) appears meaningful in this context.

These hypotheses are not necessarily mutually exclusive. Of course, there is also a possibility that the lysozyme homologues do not have bacteriolytic activities, having gained some unknown functions. Future studies should focus on the biochemical properties, substrate specificity, and antimicrobial spectrum of the bacteriocyte-specific lysozyme homologues.

Host–Buchnera transport in the bacteriocyte

Located at the host–*Buchnera* interface, the bacteriocyte is expected to be involved in exchange of various metabolites, substrates, and other molecules between the host and *Buchnera*. In agreement with the expectation, it was revealed that a number of genes for transport are strikingly upregulated in the bacteriocyte (Figure 8.2C).

The aphid–*Buchnera* mutualism is principally based on the provision of essential amino acids from *Buchnera* to the host (Baumann et al., 1995; Douglas, 1998). The upregulation of the gene encoding cationic amino acid transporter, which is involved in the import of cationic amino acids such as lysine (an essential amino acid) and arginine (a semi-essential amino acid) from the environment into the eukaryotic cells (Hoshida et al., 1996), is intriguing in this context. In the cytoplasm of the bacteriocyte, *Buchnera* cells are encased in a membrane of host origin (Hinde, 1971; Griffiths and Beck, 1973). Transporters of this type may be located on this host membrane, enabling the transport of amino acids synthesized by *Buchnera* into the cytoplasm of the bacteriocyte.

Several genes for mitochondria-related transporters (ADP/ATP translocase [a translocator that exchanges ADP and ATP across the mitochondrial inner membrane], mitochondrial oxaloacetate transport protein [a mitochondrial inner membrane protein that transports oxaloacetate and sulfate], ATP synthase subunit c [a component of mitochon-

drial ATP synthase], and mitochondrial glutamate carrier [an integral membrane protein involved in the transport of glutamate across the inner mitochondrial membrane] were also significantly upregulated in the bacteriocyte. The abundance of their transcripts may reflect high mitochondrial activity in the bacteriocyte, where active ATP synthesis and energy transfer are required for energy-consuming amino acid metabolisms. Indeed, electron microscopic studies have identified a dense population of mitochondria in aphid bacteriocytes (Hinde, 1971; Griffiths and Beck, 1973). The genome of *Buchnera* lacks most genes for TCA cycle, whereas complete gene sets for glycolysis and respiratory chain are retained (Shigenobu et al., 2000). Because TCA cycle operates in mitochondria, though speculative, the upregulated mitochondrial activity and transport in the bacteriocyte might be relevant to cooperative metabolic interactions between *Buchnera* and the organelle.

One of the upregulated genes encoded Ras-like Rab GTPase, which regulates vesicular transport of proteins and lipids between compartments in eukaryotic cells (Zerial and McBride, 2001). Because *Buchnera* cells are encased in a host membrane, intracellular trafficking mechanism of this type may play important roles in the symbiotic system. The genome of *Buchnera* lacks genes for phospholipid biosynthesis, implying that *Buchnera* is unable to synthesize its own cell membrane (Shigenobu et al., 2000). Phospholipids of host origin might be delivered to *Buchnera* cells by using the vesicular transport system.

Lateral gene transfer?

In the bacteriocyte transcriptome, two transcripts showed significant similarity only to prokaryotic genes, but not to those of *Buchnera* (Nakabachi et al., 2005). R2C00193 (10 clones) and R2C00214 (4 clones) matched to RlpA (rare lipoprotein A) precursor of *Yersinia pestis* ($E = 7.3E-11$) and a hypothetical protein of *Wolbachia pipientis* ($E = 7.1E-22$), respectively (Table 8.3). Southern blot analysis confirmed that these transcripts have corresponding loci in the aphid genome. Although genes that appear to be transferred from bacteria (especially *Wolbachia*) to insect genomes have been found in various lineages of insects (Kondo et al., 2002; Daimon et al., 2003; Hotopp et al., 2007; Nikoh et al., 2007), high level of expression of these genes in the bacteriocyte is interesting in the context of its obligatory interdependency with the mutualist, *Buchnera*. Further studies are being pursued to determine the role of these genes.

Conclusion

Whole genome analysis of *Carsonella* has yielded new insight into bacterial evolution. The genome size reduction, the AT-richness, and the retention of specific biosynthetic pathway for nutrients required by the host are common features in the genomes of bacteriocyte-restricted primary symbionts, but the *Carsonella* genome is far more drastic than any studied to date. The gene inventory seems insufficient for most biological processes that appear to be essential for bacterial replication and growth, strongly suggesting that the host bacteriocyte may compensate for these processes. Thus, it might not be far-fetched to conjecture that, as in the case of organelles, some genes were transferred from the genome of the ancestor of *Carsonella* to the genome of the ancestor of psyllids, and that they are now expressed under control of the host nucleus. Also, the genome analysis of *Carsonella* got rid of the preconception that there are limits for genome streamlining, which would lead to discoveries of many more examples of intermediates between bacteria and organelles. Indeed, the whole genome analysis of *Sulcia* of the glassywinged sharpshooter

has recently demonstrated that its size is 245 kb, corresponding to the next smallest genome of a cellular organism following *Carsonella* (McCutcheon and Moran, 2007).

Transcriptome analysis of the aphid bacteriocyte demonstrated that a number of host genes that are related to amino acid metabolism, antibacterial activity, and transport are highly expressed in the bacteriocyte. The upregulation of genes for amino acid metabolism confirmed and extended physiological data indicating that *Buchnera*-mediated production of essential amino acids from nonessential ones is the pivotal process in the symbiotic system. These results profoundly enriched our understanding of the complementary metabolic features that underpin the integrity of the host–symbiont relationship. The bacteriocyte-specific lysozyme homologues provided promising candidates that might be involved in the control and maintenance of the bacterial flora in the bacteriocyte. The upregulated genes for transport highlighted an important aspect of the bacteriocyte that mediates exchange of various molecules at the host–symbiont interface. Highly expressed genes that could be transferred from symbiotic bacteria (not necessarily *Buchnera*, though) to the host genome were also identified, which fuels the interest in the case of lateral gene transfer in the psyllid–*Carsonella* system, where the streamlining of the symbiont genome is far more extreme.

Whole genome sequencing of the pea aphid is ongoing (<http://www.hgsc.bcm.tmc.edu/projects/aphid>), in combination with a new EST project based on a normalized full-length cDNA library (Nakabachi et al., in preparation). Such genomic resources will give us comprehensive information on gene inventories of both host and *Buchnera*, greatly promoting studies on the insect–primary symbiont interaction.

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chapter nine

Endosymbionts of lice

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Introduction

Lice belong to the larger, monophyletic assemblage or proposed superorder of the Paraneoptera encompassing the Psocoptera (psocids or booklice, barklice and barkflies, and formerly Corrodentia), Phthiraptera (true lice), Thysanoptera (thrips), and the Hemiptera (true bugs including, among others, leafhoppers, treehoppers, cicadas, aphids, scale insects, whiteflies, psyllids, spittlebugs, and ground pearls). Increasingly, the psocids and true lice are grouped together as Psocodea opposite the Condylgnatha, which comprise the thrips and true bugs. Psocoptera and Thysanoptera are considered the more basal orders of the two groupings. Psocodea encompass more than 10,000 species almost equally divided between booklice and barklice, and true lice. The psocodeans are the only insects that can absorb water vapor in the adult stage, which may have preadapted them to invading habitats where access to water is a scarcity (Grimaldi and Engel, 2005). The number of psocopteran species might be underestimated because of their limited economical importance compared to true lice. On the other hand, all lice species are parasitic and as the only insect taxon, parasitic at all life stages. In 1945, already 54% of the families and 67% of the genera of all mammals had been reported extinct (Simpson, 1945; Grimaldi and Engel, 2005). Since then, many more new species of mammals have been described from fossils than from living specimens (McKenna and Bell, 1997). Considering the host specificity of lice, the majority of lice species and genera may have been lost as well. An understanding of the host phylogenesis is important for unraveling the evolution of parasitism and endosymbiosis in lice.

Three suborders have generally been recognized within the Psocoptera: the basal Trogiomorpha (barklice), the Troctomorpha (barklice and booklice), and the derived Psocomorpha (barklice). From within the Troctomorpha, true lice have arisen twice, making the Troctomorpha and Psocoptera polyphyletic (Johnson et al., 2004; Murrell and Barker, 2005). With the barklice family Sphaeropsocidae as a sister group, the lice suborders Ischnocera, Rhynchophthirina, and Anoplura form a monophyletic clade. More derived in terms of molecular evolution of the nuclear small ribosomal subunit 18S DNA gene and with the booklice family Liposcelidae (Liposcelididae) as a sister group, the fourth lice suborder Amblycera evolved independently. However, morphologically Amblycera are considered as the most basal suborder of the true lice. The oldest liposcelid species, *Cretoscelis burmitica*, is preserved in Burmese amber that dates back 100–110 million years. The Liposcelidae and true lice diverged possibly in the earliest Cretaceous 145 Ma or even earlier in the Jurassic 145–161 Ma, in which case the first hosts of Amblycera lice would have been early mammals, early birds, and possibly other feathered theropod dinosaurs as well as haired pterosaurs (Grimaldi and Engel, 2006). The oldest records of living groups of Psocoptera are wings of putative tropical barklice, Amphientomidae, and common barklice, Psocidae, from the late Jurassic of Karatau, 152 Ma (Grimaldi and Engel, 2005). The Amblycera and Liposcelidae are further separated from the other true lice by the family of thick barklice, Pachytroctidea. The molecular polyphyly of the Phthiraptera is underpinned by morphological characters of the male genitalia (Yoshizawa and Johnson, 2006). However, there remains strong support for a monophyly of lice (Grimaldi and Engel, 2006). In the past, Ischnocera, Rhynchophthirina, and Amblycera have been grouped as the chewing or biting lice or Mallophaga, and contrasted with Anoplura as the sucking lice. It is now well accepted that chewing lice evolved separately in the Amblycera and in the Ischnocera.

Mycetomes in Psocoptera

Psocids are microbial surface feeders. Toothed laciniae enable them to scrape spores, fungal hyphae, lichens, algae, and films of yeast or bacteria from the surfaces of living and decaying plants. Typical habitats are found under bark and stones, on leaf litter, in galleries of wood-boring insects, in caves, and in nests of paper wasps and bees, termites, ants, and vertebrates (Grimaldi and Engel, 2005). With such a rich diet, primary or nutritional endosymbionts are not expected. Obligate nutritional endosymbionts are normally catered for by the host through the provisions of specialized organs to hold the vitamins, cofactors, or amino acid-providing bacteria or, less common, yeasts. These mycetomes often exhibit a prominent structure. Not long after the introduction of microscopes, the first mycetomic structure was described by Robert Hooke in 1664 as the liver of human lice (Hooke, 1665). Shortly afterwards, in 1669, Jan Swammerdam illustrated details in the louse mycetome that represented the primary endosymbionts (Swammerdam, 1737). However, it took until 1919 before these structures were recognized by Sikora and Buchner, independently of each other, as mycetomes and were associated with endosymbiosis (Sikora, 1919; Buchner, 1920). Unusual structures that could represent mycetomes had not been described in the anatomical literature for Psocoptera. Only in 2006, elaborate mycetomes have been discovered in two parthenogenetic booklice and barklice species (Perotti et al., 2006). The two species have paired mycetomes situated between the midgut and ovaries. They were built of 4 to 8 big and 8 to 12 small, nucleated, rhomboid mycetocytes. The mycetomes were held together by a thin but dense, uniform, anucleate layer. Paired mycetomes in the body cavity as in these booklice are very rare in Phthiraptera. Only the male lice of elephants, *Haematomyzus elephantis* (Rhynchophthirina), contain mycetomes between the testes and

the ventral hypodermis in between fat body lobes (Ries, 1931). Booklice have a paired mycetome formed by uninuclear mycetocytes and surrounded by an anuclear membrane. Such structures have been detected in an unpaired form in the rat louse *Polyplax*, the dog louse, *Linognathus*, and in dipteran bat flies, whereas the paired form has been described in hemipteran whiteflies and the leafhopper *Cicadella viridis* (Cicadellidae).

The two psocid species are quite distant to each other. *Cerobasis guestfalica* belongs to the family of granary booklice, Trogiidae, in the basal suborder Trogiomorpha. Its habitat is very varied; it can be found on deciduous branches, conifer and evergreen branches, tree trunks, haystacks, litter under hawthorn hedges, marram grass, palings, straw bales, walls, under dead birch bark, and under stones (New, 1987; Grimaldi and Engel, 2005). Dietary components of arboreal psocids like *C. guestfalica* fall into three categories: algae like *Pleurococcus* (Chaetophoraceae) and algal components of lichens, fungi including fungal components of lichens and small amounts of substrate material such as bark flakes, or, more sporadically, foods such as pollen grains (Turner, 1975). *C. guestfalica* is quite unique among the Psocodea for its reproductive plasticity. On rare occasions a form of life-bearing, ovoviviparity, through development inside the egg inside the mother is observed (Jentsch, 1936). A very few psocid species are truly viviparous.

The second species, *Liposcelis bostrychophila*, belongs to the familiar family of booklice, Liposcelidae, which originally was part of the suborder Troctomorpha. It is notorious for its feeding on mold and glue of damp books and papers and pinned insect specimens. This species is not limited to human habitation. Like the approximately 100 described species in its family, the natural habitat of *L. bostrychophila* reaches outside decaying bark and leaf litter. *L. bostrychophila* has also been found among the fur of Asian rats and African tree mice and in the nests of cliff swallows in America and weaverbirds in Africa. The most interesting association is with the small African primate or bush baby *Galago* (Grimaldi and Engel, 2005). While some liposcelid species developed a predilection for warm-blooded vertebrates, others started feeding on insect eggs, a trait not found otherwise in the Psocoptera. *L. bostrychophila* is diploid. Sex determination in sexual relatives is of the X0 type.

The dietary requirements of *C. guestfalica* have not been experimentally investigated, but *L. bostrychophila* is easy to culture on natural and synthetic foods (Broadhead and Hobby, 1944a, 1944b; Green and Turner, 2005). *L. bostrychophila* seems to have an interesting variety in its diet. Under natural conditions, about 5% of the eggs produced by *Xyletinus peltatus* (Anobiidae) or the Virginia creeper deathwatch beetle were eaten by *L. bostrychophila* and, under experimental conditions, the eggs were eaten whether or not any alternative food was available (Williams, 1972). Psocids are also known to eat their own eggs, especially if infertile, and have been recorded eating psocid corpses and exuviae (New, 1987). *L. bostrychophila* possesses considerable cellobiase activity in the midgut and less in the foregut, hindgut, and ventral labial glands. Cellulase is only found in the midgut (Sinha and Srivastava, 1970; New, 1987). *L. bostrychophila* also feeds equally well on bacteria as on fungi (Kalinovic et al., 2006).

We assume that under natural conditions, both species do not suffer from an impoverished diet requiring supplementation by primary endosymbionts. This makes these psocid mycetomes the first mycetomes in arthropods that are not associated with obligate nutritional symbiosis. Both species harbor as sole symbionts two disparate and new members of the genus *Rickettsia* (Rickettsiaceae, α -Proteobacteria). The *Rickettsia* are obligate for early development of the oocytes and egg laying (Perotti et al., 2006). Curing the psocids stops reproduction. The *Rickettsia* might play a role in restoring diploidy in the oocytes during parthenogenetic reproduction.

An electron microscopic study on the pharynx of *Liposcelis divinatorius* recognized numerous Gram-negative bacteria in unaffected esophageal epithelial cells and in the sub-esophageal ganglion cells (Chapman, 2005). We consider this species to be *L. bostrychophila*. By its morphology, the bacteria could either be *Rickettsia*, *Wolbachia*, or both. We found a similar distribution of *Rickettsia* in nerve cells and high levels of infection in the ventral subepidermis or hyperdermis of *L. bostrychophila* (Perotti et al., 2006). The distribution and intensity of *Rickettsia* in *L. bostrychophila* resembles that of the primary endosymbionts in the cattle and pig lice *Haematopinus eurysternus* and *H. suis* (Ries, 1931).

An obligate relationship of an endosymbiont with oogenesis of the host has been described for the parasitoid wasp *Asobara tabida* (Braconidae) and one of its three *Wolbachia* strains (Dedeine et al., 2003). Wasps are haplodiploid and psocids are diplodiploid. *Wolbachia* also has an obligate relationship with sexual filarial nematodes, which are diplodiploid. The nature of the dependency is not known. *Wolbachia* is also found in the collembolan *Folsomia candida* (Isotomidae), which is diplodiploid and parthenogenetic. The role of *Wolbachia* remains elusive. Curing experiments in this species have remained unsuccessful so far (Riparbelli et al., 2006). The wide distribution and persistence of *Wolbachia* in this species favors more of a causal function during oogenesis than an opportunistic presence in a parthenogenetic species. *Folsomia* has not been searched for mycetocytes or mycetomic structures.

Parthenogenesis is common in Psocoptera (Mockford, 1971; Nokkala and Golub, 2006). Parthenogenesis is rare in Phthiraptera. The cattle biting louse *Bovicola bovis* (Trichodectidae, Ischnocera) exhibits parthenogenetic reproduction. Human head lice in particular, but most likely many species in the entire order show marked female-biased sex ratios, which is usual for haplodiploid species but practically without precedence for diplodiploid species like lice (Perotti et al., 2004a). It is not impossible but unlikely that similar mycetomic structures evolved *de novo* in two unrelated psocopteran species that are both parthenogenic. This suggests that mycetomic structures should be found in some of the sexual species of the Psocoptera as well. If this is the case, then the ancestral function of the mycetomes was to hold nutritional symbionts after all. Our assumptions about which diets will require symbiotic supplementation might need to be refined.

Our current assumptions are that nutritionally poor diets require addition or enhancement from symbiotic bacteria or yeasts. An evolutionary reconstruction proposes that phloem sap and blood have become nutritionally deficient to limit exploitation of plants and animals by phloem sap and blood-sucking insects upon which the insects then adopted supplementation by symbionts. This might certainly be the case for many species. An additional hypothesis might be that a species loses its ability to utilize or uptake certain essential nutritional factors from a rich diet. Should such a loss happen early in a lineage, obligate symbiosis might become widespread if uptake is a limiting factor and patchy if utilization is the problem. A deficiency in utilization or uptake of nutrients from a diet and subsequent compensation through symbiosis might have preadapted some lineages to explore nutritionally poor diets. This would provide a scenario where nutritional symbiosis is already in place before a switch from a free-living to a parasitic life style and diet takes place. The nutritional symbiosis has become a prerequisite for ecto-parasitism.

A partial example for such a host-symbiont interaction can be found in the case of the human head and body lice. Human lice depend completely on nicotinamide or vitamin B3 being supplied by their primary endosymbiont. This is not because blood does not contain sufficient amounts. In fact, human blood contains double to fivefold the amount required by the louse. Almost all of it resides in the blood cells. Unlike some other blood-sucking insects, human lice are practically unable to lyse human blood cells and therefore cannot utilize nutrients present in their diet. It is tempting to assume that the ancestral lice

might have had the ability to lyse blood cells and the genes for the enzymes responsible might have been lost. All extant phthirapteran lice are parasitic. This means that for the last 160 million years the genomes of lice species must have undergone profound genome reduction characteristic for parasites. Human body and head lice have indeed the smallest genomes of any hemimetabolous insect reported to date (Johnston et al., 2007). The sizes are just above 100 Mb with most of the genomes noncoding. The loss of the genes for lysing blood cells might have been a consequence of the genome reduction due to the parasitic life style. In the end, the parasitic life style of lice enforces or even initiates obligate nutritional endosymbiosis.

Our hypothesis here is that *Rickettsia* are obligate or primary symbionts of parthenogenetic Psocoptera enabling reproduction of their hosts and have secondarily overtaken mycetomic provisions originally evolved for nutritional symbionts. This view is corroborated by several anomalies in the interaction between the obligate symbiont and its host. The oocytes in booklice are infected both through the germ line and through the nurse cells. Reproductive parasites are mainly transmitted through the germ line, whereas primary endosymbionts are transmitted via secondary tissues like nurse cells. *Rickettsia* are found in three different types of tissue. *Rickettsia* are housed in differentiated somatic tissues of organs, in single-cell mycetocytes, and in an organ-forming mycetome. In well-established mycetomic associations, the nutritional mycetomic endosymbiont does not infect other tissues; in booklice it does. Reproductive parasites like *Wolbachia* often infect some of the somatic tissues. Occasional duplication of one of the mycetomes and the appearance of additional Malpighian tubules might represent another transitional stage, suggesting a very recent acquisition of *Rickettsia* by the host. Fluctuating asymmetry is quite common in insects; however, organ duplications are very rare in the ontology of insects. The duplication of one of the two mycetomes might suggest that the establishment of the new organ is still experiencing genetically some instability. In most cases with centralized Malpighian infections, the nonadapted part of tubules is free from infections; in booklice it is not (Perotti et al., 2006). The *Rickettsia* in *L. bostrychophila* are both intracellular and extracellular and found in nuclei. This is another indication for a probable ongoing transition and evolution from parasite to obligate developmental symbiont. A *Rickettsia* recently described in aphids in the sheet cells of the primary mycetome and in secondary mycetocytes might be in a similar transitional stage (Sakurai et al., 2005). The aphid *Rickettsia* affects the fitness of its host. In whiteflies, *Rickettsia* have been detected inside primary mycetocytes together with the primary endosymbiont (Gottlieb et al., 2008). The booklice *Rickettsia* might be in the transition from pathogen and secondary symbiont to obligate reproductive parasite and primary symbiont; the *Rickettsia* species in aphids might be in the transition from pathogen and secondary symbiont to nutritional and primary symbiont (Perotti et al., 2006; Braig et al., 2008).

Wolbachia in Psocoptera

The first endosymbionts that have been detected in booklice and barklice were described as *Wolbachia*- or *Rickettsia*-like bacteria. Morphologically the two bacteria cannot be discriminated. If the bacteria are found in tissues other than mycetocytes or mycetomes, the identity cannot be guessed. Coccoid and bacilliform bacteria and swollen coccoid granules have been reported from the ovaries and Malpighian tubules of the booklouse *Dorypteryx pallida* (Trogomorpha: Psyllipsocidae) (Hertig and Wolbach, 1924). The bacteria are transovarially transmitted. *Wolbachia*-like endosymbionts have been observed with electron microscopy in the ovaries, oocytes, and abdominal sub-epidermal tissues of *L.*

bostrychophila (Yusuf et al., 2000; Yusuf and Turner, 2004). These bacteria have now been molecularly characterized as primary rickettsial endosymbionts (Perotti et al., 2006).

Increasingly, *Wolbachia* is molecularly identified in psocid species. Populations of several species tested negative for *Wolbachia* (Perotti et al., 2006). *Wolbachia*-infected populations of *L. bostrychophila* have been reported from the United Kingdom and Australia. The Australian populations show multiple infections with the A and B strains of *Wolbachia*. The bacteria are readily detected in booklice with the use of multiple displacement amplification (MDA) (Mikac, 2007). A B strain of *Wolbachia* has also been detected in *Liposcelis tricolor* (Dong and Wang, 2004). *L. tricolor* was then treated for 4 weeks with 1% rifampicin to remove *Wolbachia*. Crosses between *Wolbachia*-free and *Wolbachia*-infected strains exhibited lower egg production (Dong et al., 2006). Compared with the control strain, the *Wolbachia*-free strain had in the first and second generation prolonged developmental times and reduced survivorship of immature stages, as well as reduced fecundity and longevity, resulting in much smaller $r(m)$ values. Using $r(m)$ values, the fitness, relative to the controls, for *Wolbachia*-free first and second generations were calculated as 0.45 and 0.27, respectively (Dong et al., 2006; Dong et al., 2007).

Mycetomes and endosymbionts in Phthiraptera

Lice feed on the protein keratin in hair or feathers, oily secretions, and/or blood. Blood is obtained by sucking lice through vessel or capillary feeding (solenophagy), whereas chewing or biting lice engage in pool feeding (telmophagy). As such, many chewing lice are considered to have a mixed diet of keratin feeding as the norm and feeding on skin and blood pools as the exception. The existence and extent of blood feeding of chewing lice is in most cases not known or controversial.

Lice parasitize most groups of birds and mammals. Aquatic mammals like seals that come ashore on a regular basis or meet on ice have lice (Leidenberger et al., 2007). Cetaceans (whales, dolphins, and porpoises) and sirenians (manatees, sea cows, and dugongs) that remain in the water permanently are free of lice. Monotremes (platypus, echidnas), anteaters, armadillos, and bats do not support lice.

Amblycera

Amblycera are considered the most basal lice suborder. Some 1,200 species feed on birds. Another 200 species parasitize rodents, marsupials, and other mammals.

Eomenacanthus (*Menacanthus*) *stramineus* (Menoponidae), the yellow body louse of chicken, carries an uninterrupted border of *Rickettsia*-like rodlets on the chitinous lining of the crop. The folding of the crop in the area where it passes into the esophagus bears particularly large numbers of bacteria. Ries considered these *Rickettsia* symbionts and the crop formation a host provision (Ries, 1931). Buchner is ambivalent in his interpretation (Buchner, 1965). The first observation of *Rickettsia*-like bacteria in psocids also recognized a thin border of bacteria on the epithelium of the stomach of the dustlouse *Psocus* (Sikora, 1920). *Psocus* is a vernacular name for dust-associated booklice in general and not for the genus *Psocus*. These rickettsiae were extracellular and arranged in a similar positioning. The luminal infection was stable for three years in a laboratory colony of the booklice. The transmission of these rickettsiae into the stomach of human body lice failed. Human lice might have similar rickettsiae originally described as *R. pedikuli*. The identity of *R. pedikuli* is unresolved. The rickettsiae also resembled *R. melophagi*, which formed a dense border of perpendicularly arranged bacteria on the surface of the stomach of the wingless fly

Melophagus ovinus, known as sheepouse or ked. *R. melophagi* became *Wolbachia melophagi*, but molecularly it is a *Bartonella* species in the *Rhizobiales*. In a similar way, the extracellular *Rickettsia*-like rodlets of the chicken louse and *R. pedikuli* of the human louse might not be a *Rickettsia* or an α -Proteobacteria species. The *Rickettsia*-like rodlets in the chicken louse resemble in our opinion more secondary, facultative than obligate, primary symbionts.

Ries studied lice species from a further eight genera in the Amblycera with no indication of symbiotic devices in the lice or indications of symbiotic bacteria (Ries, 1931). Amblycera lice might not depend on any nutritional endosymbionts. This leaves us so far without any connection between symbiotic associations in the Liposcelidae (Psocoptera) and the chewing or biting lice in the Amblycera, which are supposed to be sister taxa. It also suggests that the diet of the chewing and biting lice in the Amblycera is not obviously deficient in any essential constituent.

Wolbachia has been detected in lice species feeding on the American white pelican, the double-breasted cormorant, and the wood duck (Kyei-Poku et al., 2005). *Eomenacanthus* (*Menacanthus*) *stramineus* of chickens and *Hohorsella lata* of domestic pigeons are infected with *Wolbachia* as well (Covacin and Barker, 2007).

Ischnocera

Ischnocera is the largest suborder of lice with more than 2,700 described species. It has as sister clades the lice suborders Rhyncophthirina and Anoplura. Most species feed on birds. A small group in the family Trichophilopteridae comprises parasites of lemurs and indriids. The basalmost family of the Ischnocera is the family Trichodectidae or mammal chewing lice, whose members feed off eutherian mammals (Grimaldi and Engel, 2005). Eutherians are placental mammals as opposed to monotremes and marsupials. Ries investigated four species of the Trichodectidae and found no indications of symbionts or mycetomic structures (Ries, 1931). Two other louse species that parasitize poultry failed to reveal any symbiotic associations, *Oulocrepis dissimilis* (Goniodidae) (Ries, 1931; Buchner, 1965; Agarwal and Saxena, 1981; Saxena and Agarwal, 1981a, 1981b), and *Lipeurus* (*Numdilipeurus*) *lawrensis tropicalis* (Philopteridae) (Agarwal and Saxena, 1981; Saxena and Agarwal, 1981a, 1981b). How basal are *O. dissimilis* and *L. lawrensis*? Or did these lice lose their symbionts?

These data again suggest that there is no link between Psocoptera and the largest louse taxon regarding nutritional symbiosis. Although the apparent lack of symbiosis in Amblycera lice suggests that feeding on keratin of feathers of birds is not inherently a reason for nutritional symbiosis, the seeming lack of symbionts in the Trichodectidae of the Ischnocera suggests that feeding on keratin of fur of mammals is neither a reason. Both examples also show that obligate symbiosis in lice originated after basal lineages had diverged and is not as old as the lice themselves.

Wolbachia has been amplified from four species of Trichodectidae feeding on cows, sheep, porcupines, and badgers (Kyei-Poku et al., 2005; Covacin and Barker, 2007).

Members of evolutionary younger lineages of the Ischnocera carry mycetomes. Host provisions for symbionts have been described for the slender pigeon louse or wing louse, *Columbicola columbae*, *Anaticola tadornae*, *Falcolipeurus frater*, *Sturnidoecus sturni*, *Turdinirmus merulensis*, *Kélerinirmus fuscus*, *Brüelia* (*Brueelia*) *subtilis*, the golden feather louse, *Campanulotes compar*, *Coloceras damicornis*, *Aegypocerus perspicuus* (all Philopteridae), and *Goniodes pavonis* (Goniodidae) (Sikora, 1922; Buchner, 1928; Ries, 1930a, 1930b, 1931, 1932a, 1932b; Buchner, 1965; Saxena and Agarwal, 1985; Fukatsu et al., 2007). The higher taxonomy of these species is still unstable. The mycetomes in these lice are very primitive in their structure. They are more aggregates of mycetocytes than true mycetocytes. The aggrega-

tion occurs in pairs on either side of the body in the abdomen beneath the hypodermis. The first development of ovarian ampullae is noticed in Ischnocera species. Ampullae are paired filial mycetomes with the sole function of endosymbiont transmission. The ampullae are delimited by a tunica to the body cavity and a structure unique to lice. No other host provides its symbionts with a secondary mycetome from which the endosymbionts can infect the oocytes.

The primary endosymbiont of the slender pigeon louse is a new species in the family Enterobacteriaceae of the γ -Proteobacteria. The louse symbiont is related to *Sodalis glossinidius*, a secondary endosymbiont of tsetse flies and the primary endosymbiont of grain weevils (Fukatsu et al., 2007).

The slender pigeon louse, together with more derived species of the Ischnocera, is also home to *Wolbachia* (Perotti et al., 2004a; Kyei-Poku et al., 2005; Covacin and Barker, 2007). *Wolbachia* sequences have been recovered from lice off pelicans, herring gulls, and pigeons.

Rhyncophthirina

The suborder Rhyncophthirina is comprised merely of three species, one species each on African and Asian elephants, on wart hogs, and on bush pigs. The Rhyncophthirina form a sister group to the Anoplura. These elephant lice have paired mycetomes that have no relationship to the gut, which is a unique feature for the Rhyncophthirina (Ries, 1931). The complexity of the mycetome is in between that of the Ischnocera and Anoplura.

The primary endosymbiont of the elephant louse is also a new species belonging to the family Enterobacteriaceae of the γ -Proteobacteria (Hypsa and Krizek, 2007). *In situ* studies still need to confirm its identity. The closest sequenced relative is a secondary symbiont of a psyllid. It is also closely allied with the primary endosymbionts of lice of domestic pigs, boar, and cattle. The hypothesis that the endosymbionts belonged to the same lineage that split together with its lice hosts around 140 Ma ago cannot be substantiated (Hypsa and Krizek, 2007). The endosymbiont lineages in the Rhyncophthirina and Anoplura must have evolved independently from free-living ancestors.

Rhyncophthirinan lice have not yet been tested for *Wolbachia*.

Anoplura

All 600 lice in the suborder Anoplura feed on mammals and are blood-sucking. All anopluran lice that were investigated have mycetomes. The mycetome provisions can be very elaborate (Ries, 1931; Buchner, 1965). Four different lineages of apparent primary endosymbionts have been uncovered so far.

The first lineage of primary endosymbionts entails lice of domestic pigs, boar, and cattle. They have a new species in the family Enterobacteriaceae similar to the lice of elephants and a secondary symbiont of a psyllid (Hypsa and Krizek, 2007). *In situ* experiments need to be performed on the lice. The pig louse is also infected with *Wolbachia* (Perotti et al., 2004a; Kyei-Poku et al., 2005).

The second lineage of primary endosymbionts is formed by a different louse species of cattle, *Solenopotes capillatus*. Again a new species in the family Enterobacteriaceae is involved. Its closest relatives are secondary endosymbionts of the citrus mealybug and a psyllid (Hypsa and Krizek, 2007). Probes should confirm the presence of the bacteria inside the mycetomes. *Wolbachia* has not been tested for in this species.

The third lineage originates from lice of the Norway rat. Its endosymbionts are classed in the family Legionellaceae in the γ -Proteobacteria (Hypsa and Krizek, 2007). Sequenced

relatives are free-living *Legionella* species. Confirmation of *Legionella* by *in situ* hybridization is in progress. The rat louse has not yet been screened for *Wolbachia*.

Wolbachia strains belonging to the A and B supergroups have been detected in lice of dogs, coyotes, goats, cattle, and harp seals (Kyei-Poku et al., 2005). The primary endosymbionts of these lice species are not known.

The fourth lineage of symbionts is home in the lice of humans and primates. Part of the 16S gene of the primary endosymbiont of human head lice has first been sequenced by Burkhart in 2002 (Burkhart, 2002; Burkhart and Burkhart, 2006; Burckhart, 2008). The identity was later confirmed by *in situ* hybridization (Sasaki-Fukatsu et al., 2006; Allen et al., 2007; Perotti et al., 2007) and independent sequencing (Hypsa and Krizek, 2007). The primary endosymbiont of human head and body lice is now known as *Riesia pediculicola*. It is also a member of the family Enterobacteriaceae. Its closest sequenced relative is *Arsenophonus nasoniae*, a male-killing endosymbiont from which it differs by more than 10%. *R. pediculicola* occupies sequentially four different mycetomes during the development of its host, undergoes three cycles of proliferation, changes in length from 2–4 μm to more than 100 μm , and has two extracellular migrations, during one of which the endosymbionts have to outrun its host's immune cells (Perotti et al., 2007). *Riesia* and the human lice have evolved one of the most complex interactions in this nutritional symbiosis, which involves two provisional or transitory mycetomes, a main mycetome, and a paired filial mycetome. Despite the close relatedness of body and head lice, differences are present in the mycetomic provision and the immunological response (Perotti et al., 2007).

Human head and body lice are not spared from *Wolbachia* infections. European and American lice carry strains belonging to the supergroups A and B (Perotti et al., 2004a; Kyei-Poku et al., 2005; Covacin and Barker, 2007). Australian lice carry a unique strain of *Wolbachia* (Perotti et al., 2004c, 2004b). This strain has now been confirmed as a member of the supergroup F (Covacin and Barker, 2007).

Nutritional provisions of Riesia pediculicola

The dietary contribution of the primary endosymbiont to human body lice has been investigated in detail (Puchta, 1955). The requirement for symbiont-derived vitamins is different for male and female lice. The following seven vitamins are symbiont-derived and essential according to the experiments of Puchta. Minimal absolute amounts needed as a single dose per louse administered with the first bloodmeal is indicated between brackets for each vitamin. A change in the ratio of the vitamins to each other is toxic for the louse.

Thiamine (vitamin B1) [0.0058 μg]. Without thiamine, symbiont-free males can still develop to adulthood. Symbiont-deficient and symbiont-free females require thiamine. Thiamine has the least pronounced effect of the seven vitamins. Human blood contains a tenth to a fifth of the required amount.

Riboflavin (vitamin B2) [5.8 ng]. Without riboflavin, symbiont-deficient males can still develop to adulthood but not symbiont-free males. Symbiont-deficient and symbiont-free females require riboflavin for adulthood. Most larvae die during the second larval molt. Blood contains half to the full amount required, but the louse is unable to resorb it. At least half of the riboflavin resides in erythrocytes, which the louse is practically incapable of lysing.

Folic acid (vitamin B9) [8 ng]. Without folic acid, symbiont-deficient males can still develop to adulthood but not symbiont-free males. Symbiont-deficient and symbiont-free females require folic acid for adulthood. Many larvae die during the

- second or third larval molt. Blood contains about half to the full amount required. Almost all of the folic acid resides in erythrocytes, which the louse is practically incapable of lysing.
- Pyridoxine (vitamin B6) [29 ng]. Most larvae die during the second larval molt. Blood contains a twentieth to a tenth of the required amount.
- Nicotinamide (vitamin B3) [29 ng]. The third strongest effect. Most larvae die during the first larval molt. Blood contains twice to fivefold the amount required. Almost all nicotinamide resides in the blood cells. Lice are practically incapable of lysing blood cells.
- Pantothenic acid (vitamin B5) [5.8 ng as calcium salt]. The strongest effect of all. Almost all larvae die during the first molt. Blood contains a twentieth to a tenth of the required amount.
- β -biotin (vitamin B7) [0.3 ng]. The second strongest effect. Many die during the first larval molt. Blood contains about a fifth of the required amount, almost all of which in erythrocytes, which the louse is practically incapable of lysing.

Puchta excluded indirectly choline (grouped with B vitamins) and ergosterol (vitamin D2 precursor) as possible essential symbiont provisions of the endosymbiont to the louse host.

The drugstore or death-watch beetle, *Sitodrepa panicea*, and the cigarette beetle, *Lasioderma serricorne* (both Anobiidae, Coleoptera), obtain from their primary endosymbionts a similar cocktail of vitamins. Both beetles rely on thiamine (vitamin B1), riboflavin (vitamin B2), pyridoxine (vitamin B6), nicotinamide (vitamin B3), and pantothenic acid (vitamin B5) from their bacteria (Blewett and Fraenkel, 1944).

Medical implications of endosymbionts of human lice

Infestations of head lice among schoolchildren are increasing dramatically as resistance to the insecticides used in shampoo-based treatments becomes more common. The identification of the endosymbiotic bacterium of human head and body lice might facilitate the development of antibiotic treatments as a supplement to conventional pediculosis treatments based on the neurotoxicity of insecticides to kill lice. It is likely that this new, highly modified endosymbiont of lice, *Riesia pediculicola*, lacks the ability to rapidly evolve resistance to antibiotic agents, and therefore represents a viable target for treatment with antibiotics that have lost their clinical value.

Lice have been with humans since the earliest times. Treatments for head lice are widely available; nevertheless, the prevalence of lice in developed countries seems to increase. Roberts and Burgess report that in a random sample of primary schoolchildren from Wales more than 1 in 10 were infected, whereas in a UK study 58% of 7- to 8-year-old schoolchildren were found infested (Roberts and Burgess, 2005). In developing countries attack rates are even higher, with prevalences over 50% of the general population. In the United States, pediculosis capitis is estimated to affect 6 to 12 million people per year. Lice infestations are an underestimated worldwide problem that crosses all social and demographic boundaries. Lice infestations can also lead to unexpected conditions such as cervical lymphadenopathy or corneal epithelial keratitis. Body lice are important vectors for louse-borne relapsing fever, trench fever, and epidemic typhus, especially among refugees. Typhus, for example, may be brought home by foreign troops returning from endemic regions such as the former Yugoslavia. Typhus might also be obtained from a reservoir host like American flying squirrels. In big cities of developed countries, body lice are increasingly important for the transmission of urban trench fever in homeless people.

Recent experiments with artificially infected lice suggest that the disease spectrum that human body lice can vector might be much wider than is currently known (Houhamdi et al., 2006; Houhamdi and Raoult, 2006a, 2006b).

Treatment of human head lice is plagued by growing numbers of treatment failures due to the emergence of insecticide-resistant and cross-resistant lice populations, toxicity of the insecticide, and/or inherent limitations and deficiencies of the agents and procedures applied. In a suburb of Paris, France, 36.7% of the lice were homozygously resistant against pyrethroids (Durand et al., 2007). All insecticides currently in use are reliant on neurotoxicity to kill the lice at the nymphal or adult stage. The organophosphate insecticide malathion has been the most prescribed pediculicide in the United Kingdom for around 10 years (Burgess et al., 2007). Alternative classes of chemicals such as essential plant oils to repel or kill lice are limited and sometimes plagued with clinical trials producing contradictory results (Audino et al., 2007; Williamson, 2007; Williamson et al., 2007; Rossini et al., 2008). It remains to be determined whether the antibiotic effect of some essential oils especially in combination with other compounds might successfully impair the endosymbionts of lice at a very early embryonic stage (Rosato et al., 2007; Bakkali et al., 2008). The need for new pediculicides is so great that more and more oral antiparasitic agents such as ivermectin, albendazole, levamisole, and thiabendazole are considered in the fight against lice (Namazi, 2001, 2003; Akisu et al., 2006; Foucault et al., 2006). Some clearance of head lice also occurs as a side effect of mass treatment campaigns for the control of river blindness (Anosike et al., 2007). Ivermectin is now also available as a topical formulation to kill permethrin-resistant head lice (Strycharz et al., 2008).

The obligate endosymbiont embodies a new, specific target for the development of novel antilouse agents, especially for cases where classical treatment regimes fail. This is underpinned by the fact that pragmatic trials with a randomly chosen antibiotic that was orally administered showed promising effects. Shashindran and colleagues discovered by accident that a 12-year-old girl treated with co-trimoxazole (trimethoprim and sulfamethoxazole) for a bacterial upper respiratory infection also was freed of a head lice infestation without any external antipediculosis intervention (Shashindran et al., 1978). Burns proposed that the antibiotic was killing the symbiotic bacteria in the gut of the louse (Burns, 1987). The stomach disc is very closely situated to the midgut of the louse but there is in fact no connection between the mycetome and the gut lumen. The bacteria stay permanently inside the body of the louse and are transovarially transmitted. This distinction is important because the chosen antibiotic has to cross the midgut to the hemolymph and then the wall of the mycetome to the bacterial chambers. Other antibiotics might be much more efficient at that and should be systematically tested (Morsy et al., 1996). Hipolito and colleagues recommend the dual therapy of 1% permethrin as a cream and co-trimoxazole orally in cases of multiple treatment failures or cases of suspected louse-related resistance to therapy (Hipolito et al., 2001). It is important to note that this endosymbiotic bacterium is not infectious and does not have an extracorporeal stage. This means that it should not have been subjected to any exchange of antibiotic resistance mechanisms common to free-living bacteria. The only exchange with the environment might be through bacteriophages, which so far have not been implicated in vectoring antibiotic resistance. The clinical implication of this might be that the obligate louse bacteria are still susceptible to antibiotics that have completely lost their therapeutic value. The accidental discovery that the major parasitic nematodes of humans carry *Wolbachia* as the obligate symbiont has revolutionized the antibiotic treatment of elephantiasis caused by Bancroftian and Brugian filariasis and blindness caused by onchocerciasis (Hoerauf et al., 2000; Taylor et al., 2005). Aside from the medical importance, lice endosymbionts might also support investigations

into the evolution of humans; this ranges from the origin of body lice and the emergence of human clothing to the dispersal of *Homo sapiens* and its probable physical and possible sexual contact with *H. erectus* (Kittler et al., 2003; Reed et al., 2004; Raoult et al., 2008).

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Symbiotic Rickettsia

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Introduction

Rickettsia may be considered the epitome of infectious, pathogenic bacteria. High morbidity and mortality, environmental stability, low infectious dose, and persistence in infected hosts have contributed to *Rickettsia prowazekii* being weaponized as a biological warfare agent (Azad, 2007). Rickettsiae have also been implicated as the origin of the quintessential end product in the evolution of symbiosis and inheritance of bacteria, the mitochondria. *Rickettsia* seemed to be characterized by two extreme phenotypic manifestations, with an absence of any representatives in between; rickettsiae as infectious pathogens and rickettsiae as inherited and obligate organelles of eukaryotic cells. Only recently has the genomic sequencing of pathogenic *Rickettsia* allowed insight into the natural history of the pathogenic arm. The discovery of *Rickettsia* that are obligatory symbionts of their insect hosts has demonstrated that *Rickettsia* do indeed span the whole spectrum of host–pathogen associations from symbionts without which a host cell cannot survive to pathogens that will kill their host cells readily. The term *symbiosis* denotes here a long-term association that renders the symbiotic bacterium noninfectious for all practical purposes, but which in itself does not connote any beneficial or detrimental qualities.

Rickettsia is a genus of obligate intracellular bacteria in the family Rickettsiaceae and the class α -Proteobacteria or, historically, the alpha group of the purple bacteria. They are Gram-negative and non-spore-forming. Infectious bacteria are often surrounded by a microcapsule composed of protein and a slime layer. Following the release from phagosomes, rickettsiae grow freely mainly in the cytoplasm, dividing by binary fission. Rickettsiae are not surrounded by a vacuolar membrane inside the cell. Axenic cultures are not possible but many *Rickettsia* species can be propagated in chicken embryos, mammalian cell lines, and tissue cultures derived from ticks. Only recently, insect cell lines have been available too. Early on, the pleomorphic nature of *Rickettsia* had become a defining characteristic of the genus. Morphological variations range from cocci and diplococci with a diameter of 0.1 μm through

rods to filament-like bacteria 10 μm long. Many bacteria that did share pleomorphic features were originally lumped with the *Rickettsia* only to be separated later on (Kligler and Aschner, 1931; Philip, 1956; Weiss and Moulder, 1984; Dumler and Walker, 2005).

Rickettsiae do not have any flagella that might aid in mobility, but some *Rickettsia* species are far from nonmobile. They cannot actively move outside a host cell, but within and between cells, *Rickettsia*, like other intracellular bacteria such as *Listeria*, *Shigella*, *Mycobacterium*, and *Burkholderia*, can exploit the actin polymerization machinery of their hosts to induce actin-based motility (Carlsson and Brown, 2006; Stevens et al., 2006).

Together with its sister genus *Orientia*, *Rickettsia* constitute the family Rickettsiaceae. *Orientia tsutsugamushi* causes scrub typhus in humans and is transmitted by an immature stage of blood-sucking mites with their saliva. Rodents and mites are the natural reservoirs. While being transmitted horizontally to mammalian hosts by the single larval stage (chiggers), in adult mites *O. tsutsugamushi* manipulates the sex ratio of the next generation while being transmitted transovarially. Formerly, *Wolbachia*, which are symbionts of insects, mites, isopods, spiders, scorpions, and nematodes, were included as a tribe within the Rickettsiaceae. Molecular data now place *Wolbachia* in the sister family Anaplasmaceae together with *Aegyptianella*, *Anaplasma*, *Ehrlichia*, *Neoehrlichia*, and *Neorickettsia*. The new genera *Candidatus Xenohalitotis* and *Cand. Pelagibacter* will end up in families of their own.

Rickettsia as pathogens or facultative endosymbionts

The etiological agent of human epidemic, recrudescent, and sporadic typhus was the first to obtain its name in 1916 as *Rickettsia prowazekii*. It is transmitted through the feces of the human body louse, *Pediculus humanus*, or, opportunistically, of *Orchopeas howardii*, a flea of flying squirrels. Rickettsiae multiply in their insect vectors. Flying squirrels are natural reservoirs. Bacteria in the feces are scratched into a wound, mucous membrane or inhaled. It is remarkably resistant outside a host cell and can stay infectious for up to 6 months. In humans it can cause primary acute fever or stay latent for several decades and recur as Brill-Zinsser disease. Importantly, the infection is deadly for lice. The body louse is in an evolutionary sense a very young vector; it split off only around 107,000 years ago from the human head louse, *P. capitis*, when humans started wearing clothes (Kittler et al., 2003; Kittler et al., 2004). Because of the strict requirement of cloth for the body louse, this is one of the rare cases where the evolutionary history of the vector is known precisely. The ancestor to the body louse, the head louse cannot transmit human typhus.

Endemic typhus is murine typhus caused by *R. typhi* and transmitted by feces or crushed rat and cat fleas where humans are a tangential host. Rat mites and lice might be vectors as well (Reeves et al., 2006d; Choi et al., 2007). Reservoirs are rats, opossums, skunks, and cats. *R. typhi* replicates in fleas and fleas stay infectious for life but unlike the human body louse, they are unaffected by the bacteria. *R. typhi* is transovarially transmitted in fleas. *R. canada* (*R. canadensis*) has historically been grouped with the typhus rickettsiae. It has been found in several tick species but has not been clearly associated with any disease in humans or other animals. Phylogenetically, *R. canada* is not related to the typhus group rickettsiae; it forms a sister group to *R. bellii*.

Most human pathogenic *Rickettsia* belong to the spotted fever group. Major representatives are *R. rickettsii* causing Rocky Mountain spotted fever, American spotted fever, or tick typhus; *R. akari* causing rickettsialpox; *R. conorii* and its subspecies causing boutonneuse, Mediterranean, Marseilles or European, and African spotted fever, Astrakham fever, and Israel tick typhus; *R. sibirica* causing Asian tick typhus; *R. australis* causing Queensland tick typhus; *R. japonica* causing Oriental spotted fever; *R. slovaca* causing tick-

borne lymphadenopathy; and *R. africae* causing African tick bite fever. New species are being added to the spotted fever group, e.g., *R. asiatica*, *R. tamurae*, *Cand. R. kotlanii*, *Cand. R. kellyi* to name just a few (Fournier et al., 2006; Fujita et al., 2006; Rolain et al., 2006; Sreter-Lancz et al., 2006). The number of species that are recognized as human pathogens is also increasing (Owen et al., 2006). *R. heilongjiangensis* is the pathogen of the new disease, Far Eastern tick-borne rickettsiosis (Miedaniannikov et al., 2006). One of the new additions reported from Spain is *R. monacensis* (Jado et al., 2007). In all cases humans are tangential hosts. The role of wild animals or domestic animals like dogs as reservoirs or amplifying hosts is not always well understood. Also, larger animals such as deer may represent reservoirs for human pathogens such as *R. helvetica* (Inokuma et al., 2008). *R. akari* is transmitted by mites; all other spotted fever group rickettsiae are transmitted by ticks. *R. rickettsii* is vectored by numerous species belonging to several hard tick genera, whereas *R. conorii* is only transmitted by the brown dog tick *Rhipicephalus sanguineus* (Ixodidae). Tick-borne rickettsiae are transmitted by several routes to humans including salivary secretions, coxal fluids, regurgitation, and feces. *R. rickettsii* is transovarially transmitted from mother to progeny, transstadially transmitted from eggs to larvae to nymphs to adults, and sexually transmitted from male to female ticks. Venereal transmission in one generation does not necessarily lead to transovarial transmission to the next generation (Schrieffer and Azad, 1994). *R. rickettsii* is highly pathogenic to ticks, implying a major role for horizontal transmission in its maintenance; *R. conorii* is equally pathogenic to its single tick vector, indicating that vector specificity does not correlate with pathogenicity in the vector. The bacteria stay in a dormant state in the tick, avirulent to vertebrates, and require reactivation often induced through an increase in temperature due to blood-feeding before they become infectious again.

One *Rickettsia* species that is widespread in cat and dog fleas, *R. felis* (*R. azadi*), does not fit well with the classical groupings of typhus and spotted fever rickettsiae. *R. felis*, also called ELB agent, causes flea-borne spotted fever; however, the symptoms resemble more murine typhus and one of the surface antigens is shared with *R. typhi*. It does not cause any pathology in the fleas and is transovarially transmitted. Phylogenetically, *R. felis* forms a sister group to the spotted fever rickettsiae.

In addition to the classical clinical pictures of typhus and spotted fever, some *Rickettsia* infections can precipitate encephalitis in humans. *R. rickettsii* has been shown to cause apoptotic death of cultured cerebellar granule neurons (Joshi and Kovacs, 2007). Secondary neurological symptoms such as peripheral facial palsy or sensorineural hearing loss are increasingly reported (Jimenez-Caballero, 2008; Tsiachris et al., 2008).

When in the literature the vector is also described as the natural reservoir for a particular *Rickettsia* species, it mainly refers to vectors of medical importance. Ticks, mites, and insects that do not bite humans are rarely investigated. *R. felis*-like bacteria are now increasingly detected in ticks (Duh et al., 2006). *R. felis* is also found in mites (Choi et al., 2007). Indeed, many human pathogenic *Rickettsia* species can be traced to mites not only belonging to the mite family Trombiculidae that is host to chiggers transmitting *Orientia* (Reeves et al., 2006a; Reeves et al., 2007).

Individual ticks can be infected with more than one *Rickettsia* species. A *Dermacentor variabilis* tick collected in Ohio was found naturally infected with *R. bellii*, *R. montanensis*, and *R. rickettsii* (Carmichael and Fuerst, 2006). The presence of *R. felis* reduced the microbial diversity of cat flea colonies (Pornwiroon et al., 2007). *O. tsutsugamushi* appears to provide a beneficial effect against HIV infection progress, negatively influencing the virus replication process (Kannangara et al., 2005). The best studied of these interference effects is the one caused by *R. peacockii*. This species is nonpathogenic to ticks, limited to the ovaries, and

transovarially transmitted. Vertebrate infections have never been detected. Tick infections with *R. peacockii* are believed to prevent ovarian superinfection by *Rickettsia* pathogenic to ticks and pathogenic to vertebrates (Burgdorfer et al., 1981; Niebylski et al., 1997; Azad and Beard, 1998; Macaluso et al., 2002; de la Fuente et al., 2003; Baldridge et al., 2007b).

Although wild and domestic animals can exhibit high seroprevalence rates for *Rickettsia*, reports of acute clinical disease in animals are rare. Clinical symptoms in dogs, for example, are often caused by metabolic or immunologic defects associated with inbreeding (Solano-Gallego et al., 2006).

A growing number of new *Rickettsia* species are being isolated from ticks and tick cell cultures (Parola et al., 2005; Cutler et al., 2006; Mattila et al., 2007; Pacheco et al., 2007). These new species are molecularly well defined but their biology awaits a dedicated researcher to characterize. These species might be exclusive endosymbionts of their arthropod hosts or pathogens yet to be discovered as such. Some of these *Rickettsia* are vertically transmitted (Reeves et al., 2006b). *R. parkeri* was first identified in ticks in 1939, the first documentation of it in a human case had to wait until 2004 (Paddock et al., 2004). The causal agent of Q fever, *Coxiella burnetii* (Coxiellaceae, Legionellales, γ -Proteobacteria) had originally been considered as a nonpathogenic tick symbiont, *Rickettsia diaporica*. The following named rickettsial species are currently considered endosymbionts of their hosts. *R. peacockii* in the wood tick *Dermacentor andersoni* (Ixodidae) and *R. bellii* in many hard and soft-backed tick species are the best-studied, nonpathogenic species. *R. bellii* might be the most abundant and broadly distributed rickettsial species infecting ticks. *R. montana* (*R. montanensis*) in the American dog tick *D. variabilis* and *D. andersoni*, and *R. monacensis* in the sheep or castor bean tick *Ixodes ricinus* (Ixodidae) are considered not to infect any vertebrate host. Until recently, three more species were in this list, *R. rhipicephalis* in *R. sanguineus* and *D. occidentalis*, *R. tamurae* in *Amblyomma testudinarium* (Ixodidae), and *R. asiatica* in *I. ovatus*. The fact that *R. rhipicephalis* invades the salivary glands of its tick hosts makes it suspicious. An indirect immunofluorescence assay detected antigen of *R. rhipicephalis* in serum of two rural dogs from the Brazilian Amazon (Labruna et al., 2007). Dogs in Japan show high antibody titers against *R. tamurae* and *R. asiatica* (Tabuchi et al., 2007).

Rickettsia-like bacteria have been detected in several fish species but especially in salmon. Eventually described as *Piscirickettsia* species, they are allied with the γ -Proteobacteria and despite their name not related to *Rickettsia*.

Rickettsia-like organisms, RLOs, have also been associated with many plant diseases. The xylem-inhabiting RLOs have all been isolated in axenic culture and characterized as not related to *Rickettsia*. Most phloem-inhabiting RLOs have not been cultured and their taxonomic relationship remains uncertain (Davis, 1991). One of them, transmitted by psyllids and causing citrus greening, has been identified as *Cand. Liberibacter* in the Rhizobiaceae in the sister order to the Rickettsiales (Bove, 2006). The causal agent of papaya bunchy top disease is transmitted by leafhoppers and is part of the genus *Rickettsia* (Davis et al., 1998). It is an obligate intracellular bacterium of latex-producing cells. It is in the same clade as *R. bellii*. Bunchy top symptoms of papaya plants in Cuba have been associated with *Candidatus* *Phytoplasma aurantifolia* of the Achleplasmataceae in the Firmicutes, pathogenic bacteria that are also transmitted by leafhoppers (Arocha et al., 2007). The diseased papaya plants in Cuba were free of *Rickettsia*.

The association with vector arthropods of seemingly all *Rickettsia* associated with humans, terrestrial vertebrate animals, and plants is an important feature.

Members of the new genus *Cand. Xenohalictis* in the Rickettsiales are implemented in diseases associated with *Rickettsia*-like organisms of aquatic invertebrates. Foot withering syndrome is a fatal disease of abalone (Balseiro et al., 2006). *Xenohalictis* occurs in vacuoles

of the epithelial cells of digestive diverticulae (Azevedo et al., 2006). Abalones are often coinfecting with a pathogenic haplosporidian, which makes delimitation of the pathogenicity of the bacterium difficult.

Intracellular *Rickettsia*-like organisms are common in bivalves in general (Harshbarger et al., 1977; Elston, 1986), oysters (Renault and Cochenne, 1994; Wu et al., 2005), clams (Villalba et al., 1999), and decapods like prawns (Nunan et al., 2003). The identity of giant extra-cellular *Rickettsia* associated with the gill of Pacific oysters and the testes of mosquitoes remains a mystery (Azevedo and Villalba, 1991; Ndiaye et al., 1995). *Rickettsia*-like organisms reported being associated with phages hint more toward *Piscirickettsia*-like species in the γ -Proteobacteria than *Rickettsia* species in the α -Proteobacteria (Buchanan, 1978). The presence of potential pathogens and episodes of mortality are not always linked (Gomez-Leon et al., 2007). While these are mainly reports from diseased animals, many *Rickettsia*-like organisms also occur in healthy animals without any apparent detrimental effects. The lack of pathogenic changes makes the detection of *Rickettsia* a chance effect. Close endosymbiotic interactions of *Rickettsia* might be much more widespread than currently assumed. Ecological studies on microbial diversity associated with ticks return *Rickettsia* or relatives as providing the most abundant DNA sequences found in all samples (Moreno et al., 2006; Venzal et al., 2008).

Rickettsia can be reproductive parasites of their arthropod hosts (Perlman et al., 2006). Male-killing is one of the longest studied phenotypes for insect *Rickettsia*. It occurs in several unrelated beetle species including ladybirds (Hurst et al., 1993; Werren et al., 1994; Hurst et al., 1999; Hurst and Jiggins, 2000; Lawson et al., 2001; von der Schulenburg et al., 2001). Different strains of a male-killing *Rickettsia* infecting the beetle *Adalia bipunctata* are associated with distinct mitochondrial haplotypes, suggesting that the fitness of these male killers may be negatively frequency dependent or different strains may be favored in different populations (Jiggins and Tinsley, 2005). The frequency of male-killing by *Rickettsia* in beetles is very low. *O. tsutsugamushi* causes almost female-only populations in its mite hosts where it kills most males (Takahashi and Tanaka, 1995; Takahashi et al., 1997).

Induction of parthenogenesis or thelytoky by *Rickettsia* has only been recently discovered. The larval endoparasitoid wasp, *Neochrysocharis formosa*, mostly produces female progeny. Male progeny were produced by females treated with tetracycline (Hagimori et al., 2006). The *Rickettsia* infection has reached fixation in practically all populations that were studied (Tagami et al., 2006). The male-killing and the parthenogenesis-inducing *Rickettsia* form a clade together with *R. bellii*.

Many of the *Rickettsia* detected in terrestrial invertebrates are not or not yet associated with a host phenotype. *Rickettsia* have been detected in the springtail *Onychiurus sinensis*, in the red spider mite *Tetranychus urticae*, in the weevil *Kytorhinus sharpianus*, in the crane fly *Limonia chorea*, and in the biting midge *Culicoides sonorensis* (Fukatsu and Shimada, 1999; Campbell et al., 2004; Hoy and Jeyaprakash, 2005; Frati et al., 2006; Perlman et al., 2006). In the sweet potato whitefly *Bemisia tabaci*, *Rickettsia* are one of several secondary endosymbionts. Vertical transmission and biotype dependent frequency of *Rickettsia* in whiteflies suggest a phenotype that is advantageous under certain environmental conditions but may be deleterious enough to prevent fixation under other conditions (Gottlieb et al., 2006; Chiel et al., 2007).

An evolutionarily interesting association of a *Rickettsia* has been detected in a new species of amphizoic amoeba that lives in the gills of the freshwater roach (Dykova et al., 2003). The amoeba *Rickettsia* forms the most basal lineage within the genus in a clade that unites leech *Rickettsia*, crane fly *Rickettsia*, and the mycetomic *Rickettsia* of the psocid *Cero-basis* (Perotti et al., 2006).

All tick-borne *Rickettsia* species pathogenic to humans are transmitted by hard ticks. Ticks constitute three families in the mite order Acari. Rapidly feeding, hard-backed ticks belong to the family Ixodidae; slowly feeding, soft-backed ticks to the family Argasidae. Nuttalliellidae is a monotypic tick family that has not been investigated for rickettsiae (Hoogstraal, 1985). Rickettsiae have repeatedly been recorded from soft ticks (Rehacek et al., 1977; Philip et al., 1983; Hoogstraal, 1985; Noda et al., 1997; Cutler et al., 2006; Reeves et al., 2006b; Mattila et al., 2007). All these rickettsiae found in soft ticks are of undefined pathology, meaning that no link to any disease in vertebrates or humans has been established. Soft ticks can experimentally be infected with *R. rickettsii*. Natural infections have been reported from several soft tick species (Hoogstraal, 1985). An *R. rickettsii*-infected soft-backed bat tick, *Carios kelleyi* (Argasidae), is capable of inducing a weak immune response against murine typhus in guinea pigs, suggesting that more reports describing occasional transmission to vertebrates of pathogenic *Rickettsia* species normally associated with hard ticks might be expected (Reeves et al., 2006c). The rickettsiae commonly found in soft ticks are transovarially and transstadially transmitted. One of these rickettsiae, *R. bellii*, is also known from several hard ticks (Horta et al., 2006).

Some of the tick-borne rickettsiae, such as the *Rickettsia*-like endosymbiont Montezuma, are primarily associated with female ticks and are lost in males (Eremeeva et al., 2007). *R. peacockii*, an endosymbiont of the wood tick *D. andersoni*, is completely restricted to the interstitial cells of the ovaries and developing oocytes (Niebylski et al., 1997).

Most vertically transmitted bacterial symbionts have been described for insects. But even for insects, estimates of incidence and diversity might be far too low (Jiggins et al., 2001; Weinert et al., 2007). In a recent study, 8 out of 21 ladybird beetle species were positive for *Rickettsia* symbionts. Often insect samples are only tested for a few already well-studied symbiont taxa, which may not include *Rickettsia*. The importance of using appropriate DNA extraction protocols that remove host taxa-specific DNA polymerase inhibitors becomes evident in comparing symbiont surveys (Weeks et al., 2003; Zchori-Fein and Perlman, 2004). Sample size and population coverage are often emphasized for endosymbiont surveys. These are important for parasitic endosymbionts like sex-ratio distorters but not for the discovery of obligate endosymbionts. Studies on terrestrial arthropods and invertebrates other than insects are unfortunately very rare. In a study on spiders (Araneae), *Rickettsia* belonging to two novel clades were detected in 28 out of 122 species (Goodacre et al., 2006). Most of the spider species were also infected with *Wolbachia* and *Spiroplasma* species. Infection with more than one endosymbiont species is rather the rule than the exception.

Rickettsia as obligate and mycetomic endosymbionts

In the booklouse *Liposcelis bostrychophila* (Liposcelidae, Psocoptera), *Rickettsia*-like organisms were first observed by electron microscopy in the ovaries, oocytes, abdominal sub-epidermal tissues, esophageal epithelial cells, and subesophageal ganglion cells (Yusuf et al., 2000; Yusuf and Turner, 2004; Chapman, 2005). Yusuf and Turner performed the first molecular characterization of the intracellular bacteria (Yusuf and Turner, 2004). These bacteria have now been confirmed to be *Rickettsia* by sequencing and fluorescent *in situ* hybridization (Perotti et al., 2006). No pathology was evident in infected somatic tissues by electron microscopy. No bacteria have been seen in the salivary glands as might be expected from *Rickettsia* that are obligate for oogenesis. The *Rickettsia* in the parthenogenetic psocids *Cerobasis guestfalica* (Trogidae, Psocoptera) and *L. bostrychophila* occupy single cell mycetocytes (bacteriocytes) and mycetomes (bacteriomes, bacteriotomes). The

first association with dedicated host-provided structures becomes evident in first instar nymphs. Two large mycetocytes start to migrate into the fat body, one on each side of the body. These single cell mycetocytes are heavily infected with *Rickettsia*. During the final instar and teneral stage, infected cells aggregate to build a paired mycetome in between future ovaries and midgut (Figure 10.1). Each mycetome is put together by 4–8 big and 8–12 small mycetocytes, which become surrounded by a dense, uniform anucleate layer. A few cell-free *Rickettsia* were also observed in the hemolymph close to the mycetomes. The behavior of *Rickettsia* during early development in psocids resembles that of the yet unidentified primary endosymbionts of cattle and pig lice, *Haematopinus eurysternus* and *H. suis* (Haematopinidae, Phthiraptera).

A characteristic that *Rickettsia* share with endosymbionts of ciliates and termites is their presence and replication in host nuclei. *Rickettsia* can replicate in the nuclei of invertebrate and vertebrate host cells although the majority of bacterial replication occurs in the cytoplasm. Nuclear presence is much more common for psocid *Rickettsia* and *R. felis* than for other *Rickettsia* and *Orientia*.

Mycetomes have so far only been described for nutritional symbioses. Does this suggest a nutritional role for *Rickettsia*? A nutritional role would fit very well for *L. bostrychophila* as an ancestor to the Phthiraptera, most if not all of which have mycetomes. One might then expect to find *Rickettsia* in biting and sucking lice. Primary endosymbionts of lice all belong so far to the Enterobacteriaceae and Legionellaceae of the γ -Proteobacteria (Burkhart and Burkhart, 2006; Sasaki-Fukatsu et al., 2006; Allen et al., 2007; Fukatsu et al., 2007; Hypsa and Krizek, 2007; Perotti et al., 2007, 2008). No other indications support a nutritional angle at the moment. Mycetomes have not (yet) been described for other psocid species. Nutri-

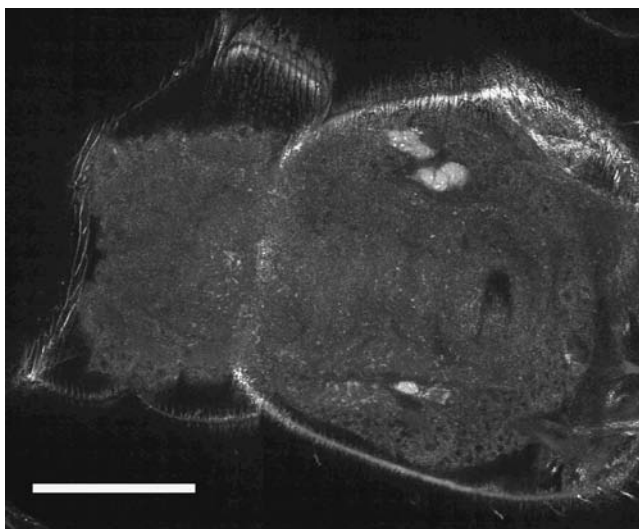


Figure 10.1 (Color figure follows p. 238.) *Rickettsia* in a paired mycetome at both sides of the body between ovaries and midgut in a teneral of the booklouse *L. bostrychophila* (Psocoptera). Ventral view of a horizontal section. Extracellular *Rickettsia* are visible in the hemolymph as well as *Rickettsia*-carrying mycetocytes in other tissues. The mycetome on the left side of the animal is duplicated. Confocal microscopy picture with a *Rickettsia*-specific probe (yellow channel). Bar 100 μ m. (Modified from Perotti, M.A., Clarke, H.K., Turner, B.D., and Braig, H.R. [2006]. *Rickettsia* as obligate and mycetomic bacteria. *FASEB J.* 20: 2372–2374 and E1646–E1656. With permission from FASEB.)

tional symbioses are not known from isolated species. If nutritional symbiosis occurs, it is spread over an entire family or even an order. So why do these two psocid species have mycetomes? No other bacteria were detected in the mycetomes. Assuming that the two *Rickettsia* of psocids are not nutritional, are we looking at the first cases where hosts lost their nutritional symbionts through a change in diet and the *Rickettsia* took advantage of the left over structures? If not, these *Rickettsia* have to be considered as organ-forming *Rickettsia* for the time being.

Evolution of obligate nutritional endosymbionts

Obligate nutritional symbioses are almost countless, especially in insects (Buchner, 1965; Moya et al., 2008). Associations can be extracorporeal and environmental like fungi and ambrosia beetles or intracellular and linked to transovarial transmission. Complex microbial communities spanning three bacterial phyla including α -Proteobacteria are vertically transmitted between juveniles, larvae, and adults by a single sponge species (Schmitt et al., 2007). Cospeciation has been reported for a range of diverse endosymbionts and hosts such as sulfur-oxidizing endosymbionts and deep sea clams (Peek et al., 1998), *Blattabacterium* (Flavobacteria) and cockroaches and the termite *Mastotermes darwiniensis* (Lo et al., 2003), *Buchnera* in aphids (γ -Proteobacteria) (Clark et al., 2000), *Cand. Carsonella* (γ -Proteobacteria) in psyllids (Thao et al., 2000), *Cand. Portiera* (γ -Proteobacteria) in whiteflies (Thao and Baumann, 2004), *Cand. Blochmannia* (γ -Proteobacteria) in carpenter ants (Degnan et al., 2004), *Wigglesworthia* (γ -Proteobacteria) in tsetse flies (Chen et al., 1999), *Cand. Nardonella* (γ -Proteobacteria) in palm weevils (Lefevre et al., 2004), *Cand. Tremblaya* (β -Proteobacteria) in mealybugs (Baumann and Baumann, 2005; Downie and Gullan, 2005), *Cand. Uzinura* (Flavobacteria) in armored scale insects (Gruwell et al., 2007), and the coprimary symbionts *Cand. Sulcia* (Flavobacteria) and *Cand. Baumannia* (γ -Proteobacteria) in leafhoppers and sharpshooters (Takiya et al., 2006). Cospeciation between endosymbionts and insect hosts might also be erroneously rejected because of particularities associated with host mitochondrial trees (Clark et al., 2000). Interestingly, most of the primary endosymbionts in the γ -Proteobacteria might have evolved independently from each other (Herbeck et al., 2005; Novakova and Hypsa, 2007). Cospeciation of nutritional symbionts does not depend on intracellular or extracellular localization or on horizontal or vertical transmission. Encapsulation of the gut symbionts in the stinkbug family Plataspididae (Heteroptera) makes horizontal transmission of an obligate nutritional gut bacterium, *Cand. Ishikawaella capsulata* (γ -Proteobacteria), as restrictive as transovarial transmission for endosymbionts. The gut symbiont exhibits AT-biased nucleotide composition, accelerated molecular evolution, and reduced genome size (Hosokawa et al., 2006). Without encapsulation, the triplex symbiosis of nutritional eukaryotic protists and their bacterial endosymbionts in the guts of termites show almost complete codivergence with the host termites (Noda et al., 2007). The purpose of this listing is not only to illustrate the diversity of associations but also to emphasize the paucity of nutritional endosymbionts in the α -Proteobacteria. The only nutritional symbiont of an animal in the α -Proteobacteria is found in leeches belonging to the genus *Placobdella* (Glossiphoniidae, Rhynchobdellida) (Siddall et al., 2004). These leeches harbor their intracellular bacteria in mycetomal organs attached to the esophagus that form a pair of pear-shaped blind sacs or caeca lined with large mycetocytes. The leeches hold vertebrate blood for digestion in these ceca. The endosymbionts, *Cand. Reichenowia*, are closely related to the nitrogen-fixing, nodule-forming Rhizobiaceae of plants, which belong to the order Rhizobiales that is like the Rickettsiales in the α -Proteobacteria. Transovarial transmission of *Reichenowia* is assumed. Other leech

families have differently styled mycetomes occupied with members of the γ -Proteobacteria (Graf et al., 2007). *Cand. Reichenowia* and the psocid *Rickettsia* are so far the only two bacteria in the α -Proteobacteria that inhabit animal mycetomes by themselves.

Two other genera, *Torix* and *Hemiclepsis*, in the same leech family Glossiphoniidae that carries *Reichenowia*, are populated by bacteria that are members of the genus *Rickettsia*. The leech *Rickettsia* are intracellular in various leech tissues such as epidermis, esophagus, and salivary glands (Kikuchi et al., 2002). The detection of the bacteria in leech eggs suggests near 100% vertical transmission for most species. The leeches *Torix tagoi*, *T. tukubana*, *Hemiclepsis marginata*, and *H. japonica* exhibit a stable infection frequency of 96, 83, 29, and 0%, respectively (Kikuchi and Fukatsu, 2005). In *T. tagoi* and *T. tukubana*, infected individuals were remarkably larger in size than uninfected individuals, whereas in *H. marginata*, infected and uninfected individuals were almost comparable in size. *Rickettsia* in *T. tukubana* mark a transition in infection frequency coupled with a nutritional benefit, one step before becoming an obligate nutritional endosymbiont and one step before host provision for symbionts. The *Rickettsia* in leeches form a sister group to the crane fly and mycetomic Cerobasis *Rickettsia*.

Aphids have mycetomes and *Buchnera aphidicola* (Enterobacteriaceae, γ -Proteobacteria) as their primary nutritional endosymbiont. They also hold several facultative or secondary bacterial endosymbionts, one of which is a *Rickettsia*, better known as PAR-symbiont (pea aphid *Rickettsia*) (Chen et al., 1996). The *Rickettsia* have a negative effect on the host fitness. This is interpreted as a probable artifact of laboratory rearing and might disappear under specific environmental conditions in the wild (Montllor et al., 2002; Sakurai et al., 2005; Simon et al., 2007). Equally possible is that this *Rickettsia* still has some pathogenicity associated with its ability to infect aphids as a new host. However, the *Rickettsia* do not seem to be easily transinfected artificially to other aphid species (Tsuchida et al., 2006). Remarkably, the *Rickettsia* in the pea aphid *Acyrtosiphon pisum* (Aphididae, Hemiptera) are not only found in the hemolymph but also in secondary mycetocytes and in the sheath cells of the primary mycetome (Sakurai et al., 2005). The amount of the primary endosymbiont *Buchnera* was significantly suppressed in the presence of *Rickettsia*, particularly at the early adult stage when the aphid host actively reproduces and has the highest nutritional demand on its primary endosymbiont. A completely opposite situation has been reported for biotypes in Israel of the whitefly *Bemisia tabaci* (Aleyrodidae, Hemiptera). *Buchnera* serves as primary endosymbiont in whiteflies as well. In *B. tabaci* the only stage at which *Rickettsia* can be seen associated with bacteriocytes is in very young embryos of eggs just having been laid; the *Rickettsia* seem to leave the bacteriocytes (Gottlieb et al., 2006). The authors now call this phenotype scattered. A second phenotype, confined, has been described for other biotypes in Israel. In confined, the *Rickettsia* are strictly localized within the bacteriocytes at all developmental stages (Gottlieb et al., 2008). The signal of the fluorescent probe is the strongest at the circumference of the bacteriocytes. This is the first case where secondary *Rickettsia* have been detected inside the mycetocytes of the primary, obligatory endosymbiont. The earlier case of *Rickettsia* compromising the replication of the primary endosymbiont in the pea aphid might have had *Rickettsia* in the primary mycetomic cells after all. *Rickettsia* were not the only secondary endosymbionts detected inside primary mycetocytes of the whitefly. *Hamiltonella*, *Arsenophonus* (both Enterobacteriaceae), *Cardinium* (Bacteroidetes), and *Wolbachia* did share the same cells with *Buchnera* in addition to *Rickettsia* (Gottlieb et al., 2008). However, *Hamiltonella* and *Arsenophonus* as well as *Cardinium* and *Rickettsia* seem to be mutually exclusive and *Cardinium* and *Wolbachia* seem to be rare in the same individual. This *Rickettsia* of aphids and whiteflies are related to *R. bellii* and the mycetomic Liposcelis *Rickettsia*. The aphid and whitefly *Rickettsia* endosymbiosis

might be the best example for the transition of a still pathogenic *Rickettsia* challenging a residing primary endosymbiont and being destined to become the first or second obligate nutritional *Rickettsia*.

The primary endosymbiont of tsetse flies, *Wigglesworthia*, has a long association with its host. Applying the dynamics of reductive genome evolution and sequence evolution to *Wigglesworthia*, both approaches suggest that the bacterium had already been a secondary endosymbiont when it colonized tsetse flies as a primary endosymbiont, so little is known yet about its early evolution toward nutritional symbiosis (Herbeck et al., 2005; Khachane et al., 2007).

When an infectious secondary endosymbiont becomes a primary endosymbiont, it is like a life sentence without parole. The primary endosymbiont loses its ability to infect. It is locked up for life; its vagility is extremely compromised. It is doomed to slowly degenerate in its genome content and in its physiological capabilities. Final death occurs when it again is replaced by another secondary endosymbiont. The evolution to “real mutualism” is only an anthropocentric illusion. There are two options to escape this scenario of annihilation.

Before degeneration progresses too far, the symbiont genes have to move to the nucleus and become functional in their new environment. The first part is rare in higher animals but no longer without precedence. A part of the *Wolbachia* genome moved to the X chromosome in the host nucleus in the adzuki bean beetle, *Callosobruchus chinensis* (Chrysomelidae, Coleoptera) (Kondo et al., 2002). The transferred genes might represent 30% of the genome of *Wolbachia* and have probably been derived from a single lateral transfer event (Nikoh et al., 2008). The genes are not transcriptional active in the nucleus. Around half of the transferred genes, 27 out of 57, have been structurally disrupted by a premature stop codon and pseudogenized, but 34 genes showed background levels of RNA in the nucleus possibly through promoter-less leaky transcription. Unequal crossing over between synapsing X chromosomes might have led to a duplication of some of the transferred genes. The transfer into the beetle nucleus might have occurred between 0.74 and 2.5 million years ago. Fragments ranging in size between less than 500 bp and more than 1 Mbp have also been detected in the genomes of three *Drosophila* species, three parasitoid wasp species, one mosquito species, and two filarial nematode species (Dunning Hotopp et al., 2007). In both nematode species, *Wolbachia* is already an obligate endosymbiont. Regaining function of the prokaryotic genes in the eukaryotic nucleus is the next important step that led nutritional symbionts to become mitochondria, chloroplasts, and apicoplasts in parasites. The first step to regaining function in the eukaryotic nucleus is being transcribed. *Wolbachia* genes are being described also in the nuclei of the salivary glands of *Anopheles* species that do not carry any *Wolbachia* bacteria (Arca et al., 2005). *Anopheles* mosquitoes transcribe *Wolbachia* genes in the nucleus in the absence of the bacterium in the cytoplasm; the beetles transcribe bacterial genes in the presence of the bacterium. However, the transcribed *Wolbachia* genes have not (yet) been shown to exert any function.

An alternative option to survive as a primary endosymbiont continuous degeneration of one's genome is the establishment of coprimary endosymbionts. This occurs in leafhoppers and sharpshooters where a Flavobacteria and a γ -Proteobacteria are both obligate as nutritional endosymbionts for their hosts (Takiya et al., 2006). The genome of the Flavobacteria *Baumannia cicadellinicola* still holds 651 genes, which is 252 genes more than *Buchnera aphidicola* (394 + 5) in the aphid *Cinara cedri*. The genome of the γ -Proteobacteria *Sulcia muel-leri* codes for only 263 genes, which is still 50 genes more than *Carsonella ruddii* (253) in the hackberry petiole gall psyllid *Pachypsylla venusta* (Psyllidae, Hemiptera) (Nakabachi et al., 2006; Pérez-Brocal et al., 2006). *S. muelleri* seem to provide the sharpshooters with several

amino acids and the cofactor menaquinone; *B. cicadellinicola* contribute the amino acids methionine and histidine that *S. muelleri* cannot provide, and many cofactors and vitamins. *B. cicadellinicola* has lost its genes for menaquinone; *S. muelleri* has lost all genes for cofactors and vitamins but menaquinone. It becomes obvious that the two endosymbionts most likely complement each other in addition to the host. The two bacteria might also reveal metabolic interdependence in the fatty acid, polyisoprenoid, and other biosynthetic pathways (McCutcheon and Moran, 2007).

Coprimary nutritional endosymbionts might turn out not to be the exemption. The secondary endosymbiont of the aphid *C. cedri*, *Serratia symbiotica*, is certainly an obligate coprimary endosymbiont. *C. ruddii* is reportedly the only symbiont in psyllids (Thao et al., 2000; Nakabachi et al., 2006). Do psyllids harbor an overlooked coprimary endosymbiont or has functional gene transfer to the host taken place? However, coprimary endosymbionts will only delay the need for either replacement by a new endosymbiont or functional transfer of the endosymbiont genes to the host nucleus. These processes apply very well to animals. The ancestor of mitochondria in animals has only lost genes to lead to a mitochondrial genome that has practically the same size and number of genes for all extant animals from insects to man. In plants, mitochondria have obtained genes through lateral gene transfer at a surprisingly high rate. Even in plastids, presumably defect genes become replaced by new bacterial homologues (Rice and Palmer, 2006).

Evolution of obligate nonnutritional endosymbionts

Obligate nonnutritional endosymbionts come in various guises and graduations. Several families of marine fish and squid employ luminous bacteria in displays that are associated with sex-specific signaling, predator avoidance, locating or attracting prey, and schooling. The host provides a dedicated organ, the light organ, to host bacteria belonging to the genera *Photobacterium* and *Vibrio* (both Vibrionaceae, γ -Proteobacteria). The light organ typically accommodates and nurtures a single light-producing species in an extracellular environment. From an immunological point of view, the symbiosis might be considered extracorporeal. Accessory tissues control, direct, and diffuse the bacterial light. There is species-specificity of the bioluminescent symbiosis in the sense that each fish or squid holds a specific bacterial species or strain. This led to proposals of coevolution of host and symbiont that might result in codivergence or cospeciation. The host selects the symbiont, which has to be acquired from the environment for each new generation. The fact itself that the transmission of the symbionts of fish and squid is horizontal and not vertical should influence the evolutionary outcome of this symbiosis. In a terrestrial environment horizontally transmitted symbionts are often limited in their dispersal options, whereas in an aquatic environment dispersal for bacteria is unhindered. This might exercise strong selection pressure on terrestrial symbionts. In the fish and squid bioluminescent symbiosis, the association is obligate for the fish and squid but not for the symbiotic bacteria. The luminous bacteria are not obligately dependent on a host for reproduction; they also can colonize other habitats like intestinal tracts, skin, and body fluids of marine animals, sediment, and even seawater. For these symbiotic bacteria no genome reduction should be expected. Phylogenetic analyses and reanalyses show that the trees for the fish and squid do not match the trees for the bacteria challenging codivergence in these obligate symbioses (Dunlap et al., 2007).

A strict species specificity of fish/squid and bacteria association would be a dangerous strategy for the host. Because the bacteria can reproduce without their hosts, there is limited selection on the bacteria to provide light and the host might end up with a bacte-

rial species that will yield less and less benefit. Certain squids and fishes have been found with two bacterial species in their light organs (Fidopiastis et al., 1998; Guerrero-Ferreira and Nishiguchi, 2007; Kaeding et al., 2007). These are seen as exceptions. We would expect that a host in general would allow low levels of competing bacteria in their symbiotic organs. When equal amounts of two species are found, that should indicate that a symbiont replacement is in progress.

The bioluminescent symbiosis in squid and fish can be regarded as a form of cyclic endosymbiosis, which requires regular reassociation events between symbiotic bacteria and host for every individual in each generation. In a terrestrial environment, cyclic endosymbiosis can be found, for example, between the fungus *Geosiphon pyriforme*, which can be found on soil surface, and the intracellular cyanobacterium *Nostoc punctiforme* (Schüssler et al., 1994). Many facultative and some obligate nutritional insect symbioses are examples of cyclic symbiosis.

A terrestrial, obligate, nonnutritional symbiosis has been discovered between a saprotrophic fungus, *Rhizopus microsporus*, and an intracellular bacterium belonging to the genus *Burkholderia* (β -Proteobacteria) (Partida-Martinez et al., 2007b). The bacterium provides the fungus with a phytotoxin, rhizoxin. A closely related strain of *Burkholderia* produces in one biotype of the fungus a highly hepatotoxic mycotoxin, rhizonin (Partida-Martinez et al., 2007a). The rhizoxin-producing strain of *Burkholderia* is also essential for the formation of sporangia and spores in the rice seedling blight fungus. Vegetative reproduction is not possible without the endosymbiont. The bacterium can still be grown axenically. Its large genome size of 3.8 Mbp suggests that it communicates with free-living populations and the association with the fungus is not yet obligate for the endosymbiont. Another newly discovered association is between the endosymbiont *Cand. Streptomyces philanthi* (Actinobacteria) in the antennae of 27 species of European beewolf digger wasps (Philanthinae, Hymenoptera) (Kaltenpoth et al., 2006). The bacterium protects wasp offspring against fungal infections. The environmental part of this bacterium is not yet investigated. An example for an endosymbiont that has just reached an obligate life style might be *Polynucleobacter* (β -Proteobacteria) in ciliates where it might compensate for a defect in glycogenolysis (Vannini et al., 2007a, 2007b).

Nonnutritional symbioses where both partners in the association are obligately dependent on each other are formed between nematodes and insects on one side and *Wolbachia pipientis* on the other side. *Wolbachia* are transovarially transmitted in nematodes and insects. Most filarial nematode species depend on intracellular *Wolbachia* for embryogenesis and larval development. Recently, *Wolbachia* was also identified in nonfilarial nematodes (Tsai et al., 2007). The genome sequencing of several *Wolbachia* strains has not yet revealed any clues about the host-symbiont interactions in filarial nematodes but a nutritional contribution seems unlikely (Heider et al., 2006). The parasitoid wasp *Asobara tabidia* harbors three different strains of *Wolbachia*. All wasp species are haplodiploid. *Wolbachia* induces thelytokous parthenogenesis in many species. One of the strains of *Wolbachia* in *Asobara* is essential for oogenesis. This strain suppresses apoptosis in nurse cells. Nurse cells normally undergo apoptosis during egg development. The hypothesis is that the host evolved to compensate for the endosymbiont-induced suppression. When the endosymbiont is removed, the host compensation might actually prevent oogenesis (Pannebakker et al., 2007). Thus parasitic inhibition of cell death would facilitate symbiosis (Aanen and Hoekstra, 2007). If *Asobara* wasps are cured of the final *Wolbachia*, extensive apoptosis of the nurse cells of mid-stage egg chambers sets in. In *Wolbachia*-infected wasps no apoptosis is observed. What is the effect of removing *Wolbachia* on the egg cell? *Wolbachia* is obligate for oogenesis in this species. If oogenesis stops in a mid-stage egg chamber because oogen-

esis stops, wouldn't the failure of oocyte development at this very stage have apoptosis as a consequence? We would like to propose that apoptosis is a consequence of failing oogenesis and is not related to host compensation. We consider this a more parsimonious explanation. *Wolbachia* in this case do not inhibit cell death and do not facilitate symbiosis. However, in individuals of this wasp species we see simultaneously *Wolbachia* strains that are reproductive parasites or pathogenic endosymbionts that might cause parthenogenesis, and that are obligate endosymbionts for oogenesis, strongly suggesting that obligate relationships evolve from pathogenic or parasitic associations. A mutant *Drosophila melanogaster* strain has also become dependent on *Wolbachia* for oogenesis. *Wolbachia* restores oogenesis in mutant females prevented from making eggs by protein-coding lesions in Sex-lethal (Sxl), the master regulator of sex determination (Starr and Cline, 2002). An overlap of two phenotypes is seen in adzuki bean borer moth, *Ostrinia scapulalis*, and a sister species, *O. furnacalis*. In a *Wolbachia*-infected strain, males selectively die during larval development, whereas females selectively die when *Wolbachia* are eliminated by antibiotic treatment (Sakamoto et al., 2007). Death in the female line occurred mainly throughout early larval stages but development to the penultimate instar was possible. *Wolbachia* is here both a male killer and a sex-specific obligate endosymbiont. Females are heterogametic in moths. In the black twig borer or ambrosia beetle, *Xyleborus ferrugineus* (Curculionidae, Coleoptera), a morphologically identified *Staphylococcus* endosymbiont and a nonidentified rod-shaped bacterium are essential for egg development (Peleg and Norris, 1972, 1973; Norris and Chu, 1980). The symbiont(s) is/are intracellular and transovarially transmitted. The beetle is haplodiploid and reproduces through arrhenotokous parthenogenesis. The date stone beetle, *Coccotrypes dactyliperda* (Curculionidae, Coleoptera), suffers a similar breakdown of oogenesis when treated with antibiotics (Zchori-Fein et al., 2006). It is also haplodiploid and reproduces through arrhenotokous parthenogenesis. The date stone beetles carry a double infection of a *Wolbachia* strain and a *Rickettsia* strain. Both are transovarially transmitted. Antibiotic treatment affects *Wolbachia* and *Rickettsia* equally. One of the two or both are obligate endosymbionts. Is one of them trying to replace the other?

Rickettsia have been found in parthenogenetic species of the barklouse, *Cerobasis guestfalica*, and the booklouse, *Liposcelis bostrychophila* (Perotti et al., 2006). Two populations of *C. guestfalica* from Wales, one from the Island of Anglesey (Ynys Môn) and one from the mainland, tested positive. Three populations of *L. bostrychophila*, one each from England, Wales, and the Czech Republic, were positive. All individuals tested of a population sample harbored *Rickettsia*. *Rickettsial* sequences were also reported for three populations in China (Wang et al., 2006). The *Rickettsia* in *L. bostrychophila* are transovarially transmitted. Removal of the bacteria stops oogenesis. The same is assumed for *C. guestfalica* as well but the proof is still outstanding because of difficulties of putting this species in culture. *C. guestfalica* belongs to the family Trogidae in the suborder Trogiomorpha, which forms a basal or primitive lineage in the booklice and barklice order Psocoptera. *Rickettsia*-like organisms have also been detected previously in another family of this suborder. Bacteria have been found in the ovaries, eggs, and Malpighian tubules of the barklouse *Dorypteryx pallida* (Psyllipsocidae) (Hertig and Wolbach, 1924). The presence of bacteria in the eggs shows transovarial transmission. However, *Rickettsia* cannot morphologically be differentiated from *Wolbachia*. *L. bostrychophila*, on the other hand, is part of the family Liposcelidae, which is considered one of the most derived lineages and is phylogenetically closer to the members of the biting and chewing lice order Phthiraptera than to the other psocids. The *Rickettsia* in the two psocid lineages are evolutionary as far apart from each other as the hosts are (Figure 10.1). Indeed, the basal *Cerobasis* hosts a *Rickettsia* that associates with equal basal *Rickettsia* isolates from crane flies, leeches, and amoeba. The closest sequenced

relative to the barklouse *Rickettsia* is currently *R. limoniae* from crane flies. The Liposcelis *Rickettsia* form a group with *R. felis* and the bacterium found in a parthenogenetic wasp species (Figure 10.2). These *Rickettsia* form a sister group to *R. australis* and *R. akari*, which are transmitted by ticks and mites.

The *Rickettsia* in the date stone beetle and the two psocid lineages should not be isolated occurrences. Several sexually reproducing psocid species have tested negative for *Rickettsia*. We should expect to find *Rickettsia* in related, sexual species. In these species, we would like to predict that the *Rickettsia* still express low levels of pathogenicity to their hosts. In *Wolbachia* and *Orientia* we have seen large numbers of ankyrins-repeat genes most likely involved in host manipulation. Should we expect an increased number of ankyrins-repeat genes also in the oogenesis manipulating, obligate *Rickettsia*?

The obligate psocid *Rickettsia* exhibit several phenotypic behaviors that can be interpreted as transitional stages in their evolution of host-parasite interaction (Perotti et al., 2006). *Rickettsia* enter the developing oocyte both through the germ line and through the nurse cells. Germ line transmission is characteristic for reproductive parasites like *Wolbachia*, whereas nutritional endosymbionts often reach the oocytes through nurse cells. In a few of the psocids examined, one of the paired mycetomes was duplicated (Figure 10.2) and additional Malpighian tubules appeared. Organ duplication is very rare during the development of insects. The duplication of these organs could indicate a still ongoing process in the host of fine-tuning recently evolved provisions for obligate endosymbionts. Primary endosymbionts only reside in mycetomes in well-established systems of nutritional symbiosis. Psocid *Rickettsia* are found not only in mycetomes but also in many other tissues.

In the obligate systems described for the beetle and booklice, the association is obligatory for both partners. The *Rickettsia* in the booklice can no longer leave their host. We would predict that the genomes of these *Rickettsia* strains should show further genome reduction than the human pathogenic *Rickettsia* sequenced so far.

Genome reduction and genome isolation are linked. There is only one possible evolutionary outcome, degradation. Because of genome reduction and isolation, obligate endosymbionts will have a finite lifespan as a species or a limited shelf life as a functional endosymbiont. The provision of the symbiont to the host will eventually degrade to a point where it might severely affect the host. In order not to go under with its symbiont, the host must allow for the eventual replacement of the symbiont. This means that the host must allow limited concurrent infections with slightly pathogenic bacteria. A bacterium has to be infectious to invade a new host species. The bacterium will be naïve to the new host and involuntarily cause some pathology. The new bacteria have to infect the host intracellularly to escape the immune system.

Evolution of *Rickettsia*

The scenario of phenotypic evolution of *Rickettsia* as pathogens in relation to their tick, mite, and insect hosts and vectors seem to be simple. The presumed old associations of spotted fever group *Rickettsia* with their tick and mite hosts and vectors are characterized by no adverse effects to the ticks, transovarial transmission of the *Rickettsia* from mother to offspring, and transstadial transmission to adults. The sexual transmission of *R. rickettsii* might be interpreted as a transitional stage to full vertical transmission or inheritance. The murine typhus *Rickettsia* also causes no known pathology in its flea host. The absence of transovarial transmission might be understood either as a younger association or as the result of potential host barriers in the flea as an insect compared to ticks such as *Acari* or mites. The definitely youngest association of *R. prowazekii* with human body lice is charac-

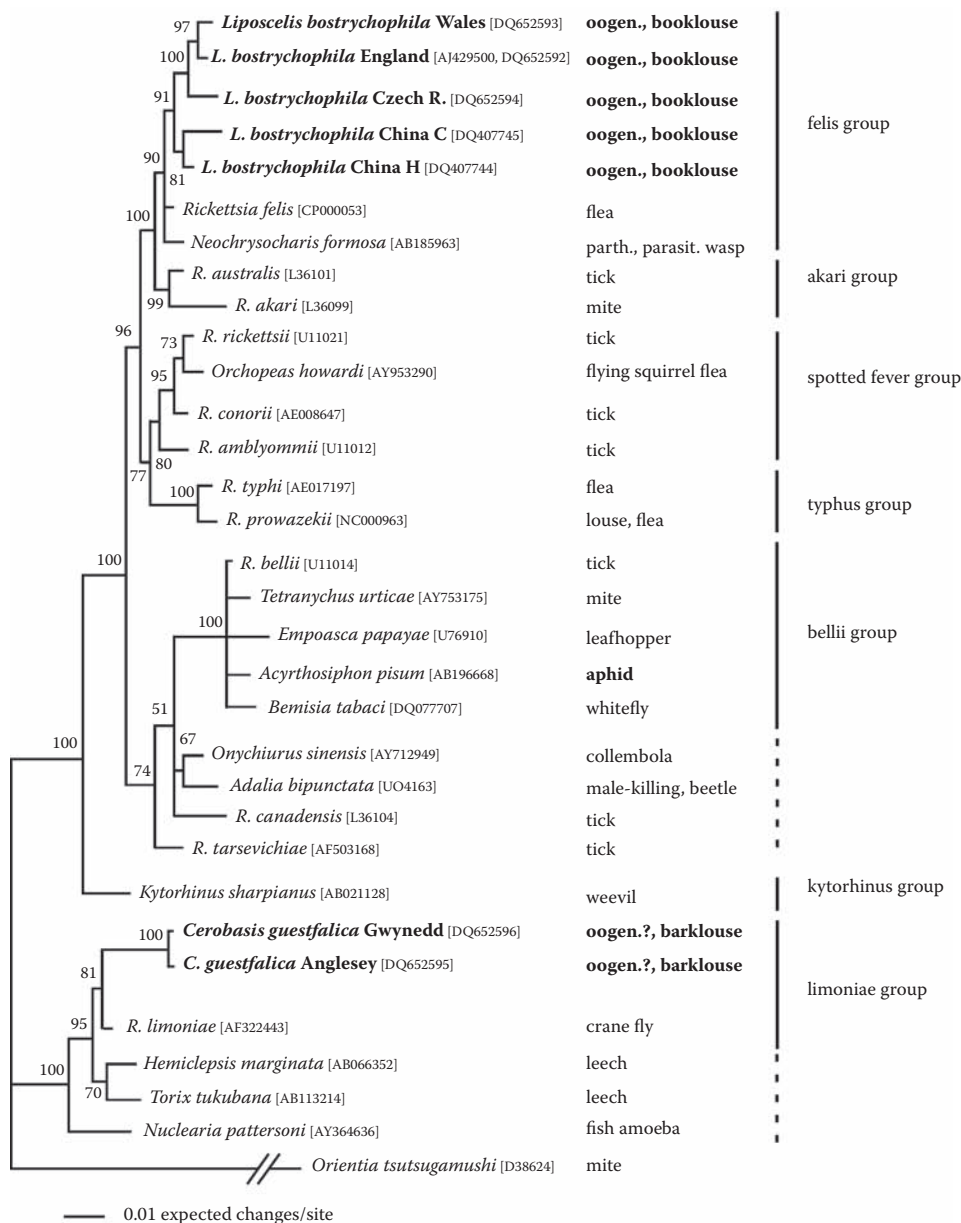


Figure 10.2 The phylogenetic positions of *Rickettsia* associated with mycetomes, oogenesis, parthenogenesis, male-killing, pathology, or of unknown phenotype. *Rickettsia* as primary endosymbionts of mycetomes are indicated with boldface. The *Rickettsia* in the aphid *Acyrtosiphon pisum* is a secondary endosymbiont of mycetocytes and mycetomes. The posterior probabilities tree is based on 16S rRNA sequences. GenBank accession numbers are given in brackets. The tree has been rooted with *O. tsutsugamushi* as an outgroup. The numbers at nodes represent clade probability values. In cases where the rickettsiae have not been formally named, the name of the host is given. Next to the name of the *Rickettsia* is a column with the dominant hosts. Conservatively, six major groups in the genus *Rickettsia* have here been identified and proposed. (Modified after Perotti, M.A., Clarke, H.K., Turner, B.D., and Braig, H.R. [2006]. *Rickettsia* as obligate and mycetomic bacteria. *FASEB J.* 20: E1646–E1656. With permission from FASEB.)

terized by complete mortality in the louse vector, whereas the flea vector of flying squirrels does not seem to be affected by *R. prowazekii*.

Several authors have proposed that pathogenic microbes transmitted by ticks arose from preexisting beneficial associations between ticks and their microbial partners (Weller et al., 1998; McFall-Ngai and Gordon, 2006; Mattila et al., 2007). Darby and colleagues generalize this hypothesis further (Darby et al., 2007).

For *R. prowazekii* and lice and *R. typhi* and ticks, this hypothesis clearly has to be refuted. We would like to propose that vector-borne *Rickettsia* become pathogens of new vertebrate hosts at the same time that the *Rickettsia* infect their new arthropod hosts. For tick-borne *Rickettsia* this would mean that they became pathogens of vertebrates when they infected their new tick hosts for the first time. The first infection of the new tick, flea, or louse host might equally be neutral or pathogenic for the arthropod. If pathogenic for the tick, selection will then start eliminating primarily the tick genotypes most susceptible to pathological effects and secondarily the bacterial genotypes most responsible for pathology in ticks.

Darby and colleagues also propose that transovarial transmission of *Rickettsia* in arthropods is the ancestral phenotype that has been lost in some genera and species (Darby et al., 2007). We would like to propose the opposite.

All *Rickettsia* species pathogenic for humans still have an animal reservoir, which makes the evolution of human pathogenicity a relatively recent event. Species like *R. canada* might still have to make this transition. Only *R. prowazekii* has a human parasite as a vector; all other *Rickettsia* species are transmitted opportunistically by animal parasites to humans and remain as such zoonoses.

The evolution of *Rickettsia* genomes is best analyzed from the perspective of *O. tsutsugamushi* that has so far the largest genome in the Rickettsiaceae with 2.1 Mbp, almost twice the size of *R. prowazekii*. It has 1,216 potentially functional genes of which it shares a core of 512 with seven other *Rickettsia* genomes (Cho et al., 2007). In addition, the genome codes for 963 fragmented genes, which most likely are nonfunctional pseudogenes. Pseudogenes are very unusual for free-living bacteria. The majority of the fragmented genes coincide with repeated DNA regions. With 4,197 identical repeats with an average length of 947 bp, the *Orientia* genome is the most highly repeated bacterial genome known so far. *O. tsutsugamushi* has 200 times more repeats than *R. prowazekii*. *Orientia* also contains 1,146 mobile elements constituting around 40% of the genome, *R. prowazekii* practically none. Prominent are genes for conjugative type IV secretion systems involved in lateral gene transfer. Of one of these genes, *tra*, *O. tsutsugamushi* has 359 copies, *R. felis* has four copies on a plasmid, and *R. bellii* just one copy on its chromosome. Tick-borne spotted fever *R. massiliae* might have obtained 14 genes for lateral gene transfer from *R. bellii* (Blanc et al., 2007). Acquisition of mobile elements might be a chance effect. The huge effective population size of free-living bacteria should prevent the expansion of mobile elements and repeated sequences in genomes. This is no longer given for obligate intracellular bacteria. Acquisition of mobile elements might have been so recent that loss had not much impact yet. Lateral gene transfer seems to be a dominant feature while recombination is quite rare in *Rickettsia* in contrast to *Wolbachia*, where the opposite situation is found (Jiggins, 2006; Baldo and Werren, 2007).

Several *Rickettsia* species also carry plasmids. *R. felis* has at least one plasmid, pRF with 64 Kbp (Ogata et al., 2005). The second plasmid, pRF delta with 39 Kbp, might be an artifact of the genome assembly (Gillespie et al., 2007). *R. amblyommii* and the nonpathogenic *R. peacockkii* carry at least two plasmids of different sizes (Baldrige et al., 2008). All other *Rickettsia* species have so far only one plasmid: *R. monacensis*, 23 Kbp, *R. massiliae*, 15 Kbp,

R. helvetica, *R. amblyommii*, and *R. hoogstraalii* (Baldrige et al., 2007a; Blanc et al., 2007; Baldrige et al., 2008). The plasmid of *R. monacensis*, pRM, is remarkably similar to the larger *R. felis* plasmid, pRF. Some of the genes on these two plasmids are otherwise found only on chromosomes of *R. felis* or the ancestral group rickettsiae *R. bellii* and *R. canada* (Baldrige et al., 2007a; Gillespie et al., 2007). The plasmid of *R. peacockkii* seems not to be essential; it was lost during long-term serial passage in cultured cells (Baldrige et al., 2008).

The presence of plasmids, mobile elements, and conjugational systems might emphasize the infectious nature of *Rickettsia* and *Orientia* and their horizontal transmission. In obligate symbionts, these features should have been lost.

Rickettsia genomes vary in size from 1.1 Mbp for the typhus group, 1.2–1.4 Mbp for the spotted fever group, and 1.5 Mbp for *R. bellii* accounting for 800–1,000 protein-coding genes. *Rickettsia* are often highlighted as having a reduced genome, especially compared with a free-living bacterium such as *Escherichia coli*, which has a genome size of 4.6 Mbp. However, if *Rickettsia* are compared with *Cand. Pelagibacter ubique*, which with 1.3 Mbp has the smallest genome of any free-living bacterium at the moment of writing, genome reduction is less impressive. Compared with obligate insect endosymbionts and pathogens such as *Wigglesworthia glossinida* in tsetse flies with 0.7 Mbp and *Wolbachia pipientis* with 1.27 Mbp in *Drosophila melanogaster* and 1.08 Mbp in the nematode *Brugia malayi*, the genomes of *Rickettsia* are still large and might undergo considerable further reduction.

Genome sequencing of *Rickettsia* has not identified any genes that might be directly responsible for overt pathology. Some virulence genes in the wider sense that code for outer surface adhesion proteins and genes involved in the infection process have been identified (Ogawa et al., 2006; Uchiyama et al., 2006). The rickettsial outer membrane protein A and member of the autotransporter family of proteins, rOmpA, is a critical protein for the adherence of *R. rickettsii* to vertebrate host cells (Renesto et al., 2006). *R. peacockii*, a close but avirulent relative to *R. rickettsii*, does not express the gene due to premature stop codons (Baldrige et al., 2004). A genomic comparison of the virulent *R. rickettsii* Sheila Smith and avirulent Iowa strain revealed 143 deletions and 492 single-nucleotide polymorphisms (Ellison et al., 2008). One of the deletions truncates rOmpA. The avirulent strain is also defective in the processing of rOmpB, both of which might contribute to the loss of virulence. The two strains display only four differences in gene expression analysis of microarrays (Ellison et al., 2008). There are no indications for any endotoxins or exotoxins entailed in pathogenesis of *Rickettsia* species. The salient pathological effect of *Rickettsia* is the destructive replication in endothelial cells resulting in vascular inflammation and hemostatic alterations. However, proteomic analysis of *R. prowazekii*, for example, detected a methyltransferase only in the virulent Breinl strain but not in the avirulent Madrid E vaccine strain where the gene has undergone a frameshift mutation (Chao et al., 2007). The vaccine strain can revert back to virulence when passaged in animals (Zhang et al., 2006).

Two interesting features in the genomes might be linked to host manipulation. Ankyrin-repeat proteins are involved in protein–protein and protein–chromatin interactions in eukaryotes; they are very rare in free-living bacteria. *Wolbachia* strains have the highest number of these genes. *O. tsutsugamushi* that like *Wolbachia* manipulates its invertebrate host harbors 50 ankyrin-repeat genes and 27 tetratricopeptide repeat genes. *R. felis* has a few ankyrins repeat genes whereas *R. prowazekii* only has remnants, suggesting loss of ankyrins-repeat genes with genome size reduction. Ankyrins-repeat genes in *Orientia*, *Rickettsia*, and *Wolbachia* are flanked by mobile and phage elements. *Anaplasma phagocytophilum* causing human granulocytic anaplasmosis and vectored by ticks translocates an ankyrin-repeat protein into the host cell where the protein becomes phosphorylated by host kinase before binding a host tyrosine phosphatase (Ijdo et al., 2007; Lin et al., 2007). A second

group of genes in *Rickettsia* genomes that sparks interest as potential host manipulators are enzymes that regulate the activation state of other proteins through phosphorylation. *Orientia* shares homologues of most of its 56 histidine kinase and response regulator domains with *Rickettsia* and *Wolbachia* (Cho et al., 2007).

Despite carrying more potentially functional genes than *Rickettsia* species, *O. tsutsugamushi* has lost more metabolic functions than *Rickettsia* (Fuxelius et al., 2007). Rickettsial genomes have lost all glycolytic genes and have to import ATP and other compounds such as pyruvate and amino acids to feed the citric acid or Krebs cycle for energy generation. The pathways for amino acid synthesis are absent or compromised. Riboflavin, vitamin B6 (pyridoxine), and nicotinamide have to be imported as well. None of the sequenced *Rickettsia* species except the nonpathogenic *R. bellii* have a complete biotin pathway (Fuxelius et al., 2007). *Rickettsia* can also no longer themselves produce nucleoside monophosphates.

Rickettsia as ancestors of mitochondria

Broadly three major groups of hypotheses have been put forward for the possible origin of mitochondriate eukaryotes. The first group postulates the endosymbiosis of an Archaea such as a *Thermoplasma*, methanogens, or a crenarchaeote (eocyte) with a Bacteria being a spirochaete, an H_2 -producing δ -Proteobacteria, or a Gram-negative bacterium, respectively. This is followed by an O_2 -consuming α -Proteobacteria such as a *Rickettsia*. The second group proposes that a Gram-positive bacterium gave rise to neomuran, the ancestor of both Archaea and current Bacteria. The bacterial arm then engulfed again an O_2 -consuming α -Proteobacteria such as a *Rickettsia*. Both groups assume an amitochondriate eukaryote as an intermediate. The fact that no ancestral amitochondriate eukaryote has ever been detected and that all extant amitochondriate eukaryotes have secondarily lost their mitochondria or still possess equivalents to mitochondria, led to the third group of hypotheses without an amitochondriate eukaryote as an intermediate step. This group presumes the endosymbioses of an Archaea directly with an O_2 -consuming α -Proteobacteria such as a *Rickettsia* or an Archaea that is either a H_2 -consuming or a H_2S producing with an α -Proteobacteria that is H_2 producing or H_2S consuming, respectively; in the latter two cases the α -Proteobacteria are not *Rickettsia*-like.

Despite a long-standing interest in the endosymbiotic origin of mitochondria (Altmann, 1980; Margulis, 1993; Sapp, 1994), no obligate endosymbiotic bacterium has been identified as a potential relative to mitochondria. Many studies place the origin of mitochondria within the order Rickettsiales (Gupta, 1995; Lang et al., 1999; Wu et al., 2004; Fitzpatrick et al., 2006) or within the family Rickettsiaceae, in particular close to *R. prowazekii* (Andersson et al., 1998; Karlin and Brocchieri, 2000; Ogata et al., 2001; Emelyanov, 2003). Alternatives to a strict *Rickettsia* lineage have been proposed; for example, the free-living and nitrogen-oxidizing bacterium *Paracoccus denitrificans* (Rhodobacteraceae, Rhodobacterales, α -Proteobacteria) (John, 1987), the free-living photosynthetic bacterium *Rhodospirillum rubrum* of the family of purple nonsulfur bacteria Rhodospirillaceae (Rhodospirales, α -Proteobacteria) (Esser et al., 2004), and the infectious bacterium *Holospira obtusa* (Rickettsiales) that invades the somatic macronucleus of the ciliate *Paramecium caudatum* (Lang et al., 2005). The latter bacterium might have a beneficial effect on its host through the supply of biotin, Hsp70 and/or GroEL, which might increase the survival of the host at low temperatures and provide survival and increased motility at high temperatures (Fujishima et al., 2005). Cavalier-Smith argues on functional reasons that the mitochondrial ancestor should have been a photosynthetic nonsulfur purple bacterium (Cavalier-Smith, 2006). Examples

of such bacteria as endosymbionts are known (Fenchel and Bernhard, 1993). It is also possible that the ultimate ancestor of *Rickettsia* could have been photosynthetic.

A concatenated alignment of 15 mitochondrion-encoded proteins that are unlikely to have undergone any lateral gene transfer in the timeline under consideration places the mitochondria inside the order Rickettsiales (Fitzpatrick et al., 2006). A species tree for 72 α -Proteobacteria produced from concatenating the members of 104 well-behaved protein families anchors the mitochondrial branch within the Rickettsiales as a sister to the combined Anaplasmataceae and Rickettsiaceae and all embraced by Pelagibacter as an out-group (Williams et al., 2007). Regardless of the lineage, in none of these cases an obligate dependence of the host has been established. The *Rickettsia* described in the two booklice species are currently the closest obligate symbionts to mitochondria. The biology of the booklice *Rickettsia* suggests that their obligate endosymbiotic lifestyle is a very recent acquisition.

Unprecedented for intracellular bacteria but common for mitochondria, the import of a host protein has been reported for *Rickettsia*. Mitochondrial porin was identified in *R. prowazekii* by Western blot analysis (Emelyanov and Vyssokikh, 2006). A rickettsial putative peptidase (RPP) of *R. prowazekii* that resembles the α - and β -subunits of the mitochondrial processing peptidase can specifically hydrolyze basic host peptides and presequence peptides with frequent cleavage at their MPP-processing sites, showing that a *Rickettsia* enzyme can cleave the signal sequences of host proteins targeted to mitochondria (Kitada et al., 2007).

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Structure and function of the bacterial community associated with the Mediterranean fruit fly

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Introduction

Insects are indisputably the dominant multicellular organisms in terrestrial habitats. As such they maintain intricate and complex interactions with the other organisms in their habitat. Some of these interactions, such as those between insects and plants, or between insects and vertebrates, have been extensively studied. On the other hand, the associations between insects and microorganisms, while pervasive and of paramount ecological importance, are relatively poorly understood. Indeed, the interactions between microorganisms and insects can be seen as a vast, unexplored region, briefly glimpsed, waiting to be charted.

In this chapter we review our work on the microflora of the Mediterranean fruit fly (medfly) *Ceratitis capitata* (Diptera: Tephritidae). We briefly introduce basic methods in molecular microbial ecology and review previous studies on tephritids and their associated bacteria. Then we describe in detail the communities we have found in the medfly,

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using culture-dependent and culture-independent techniques. We review a number of experiments aimed at deciphering the functional interactions between the medfly and its bacteria and allow ourselves a number of speculations on these interactions, which we hope will be investigated in the future.

Molecular microbial ecology

Understanding symbiosis requires identifying and characterizing the bacterial partners. This has classically been done with microscopic observations and by isolating bacteria associated with the host, further studying the isolated microorganisms, manipulating them, and reintroducing them into their host when possible. Although this approach yields thrilling results (e.g., the legume-rhizobia symbiosis), it is not always possible to characterize the molecular events occurring during the interaction of the microbial partner with its host, particularly when the microbial partner cannot be isolated. Analysis is further restricted when community structure and its diversity are the subject of the investigation. The development of methods enabling the detection, identification, quantification, tracking, and more recently whole genomes sequence analysis of unculturable bacteria has revolutionized the field of microbial ecology in general, and that of symbiosis and mutualistic interactions in particular. This quantum leap is mostly attributable to the availability of ever more sophisticated techniques that can be rapidly adopted by research laboratories. Most prominent among those are the rRNA-based technologies that use ribosomal genes (mainly the 16S rRNA gene) as phylogenetic markers. Tools based on the rRNA approach can be used, among other possibilities, to analyze population structure and to compare community patterns, for *in situ* quantification, and for studying spatial distribution at the microbial scale. In recent years the use of the rRNA approach has enabled the further characterization of the enteric microbiota of many insects (Egert et al., 2003; Reeson et al., 2003; Dillon and Dillon, 2004; Mohr and Tebbe, 2006). Excellent books and reviews covering these methods have been published (for further details see Van Elsas et al., 2007; Akkermans et al., 2004), and therefore a description of their principles is not within the scope of this chapter.

Understanding the evolution of symbiotic relationships is now within our reach: lineages can be traced back, the specificity and depth of the interaction can be determined with great precision, and impacts on genome evolution in both partners measured. A now classical protocol applied for the discovery of microorganisms associated with a host is to extract DNA from an organ or from the whole host body and to construct a 16S rDNA library based on the amplification of the target gene or parts thereof. The resulting clones can then be grouped according to the cloned sequences' restriction patterns (e.g., amplified rDNA restriction analysis, ARDRA) and representative inserts of each group sequenced or random clones sequenced. The size of each ARDRA group can be used as an indication of the distribution of the various sequences within the community. As large-scale sequencing becomes cheaper, sequencing large numbers of random clones is becoming common, yielding better information on diversity and richness (Huber et al., 2007). Based on the acquired data, specific oligonucleotides can be designed for fluorescent *in-situ* hybridization (FISH), and used for localizing and quantifying target organisms in the host's tissues. These tools have proven extremely successful, revealing hitherto unknown associations between bacteria and arthropods (Favia et al., 2007; Fukatsu and Nikoh, 2000; Kikuchi et al., 2005), deciphering modes of transmission between parents and progeny (Dobson, 2003; Kikuchi et al., 2007; Wang et al., 2004), uncovering patterns of genome evolution (Hosokawa et al., 2006), and pointing to multiple partners' symbioses (Ikeda-Ohtsubo et

al., 2007), including the occurrence of intracellular bacterial symbionts of bacteria (von Dohlen et al., 2001).

The analysis of the microbiota's community structure can also be pursued with denaturing gradient gel electrophoresis (DGGE), single-strand conformation polymorphism (SSCP), or terminal fragment length polymorphism (T-RFLP). These techniques enable population profiling of large numbers of samples in parallel, and with the proper statistical tools, community patterns can be compared (Lacava et al., 2007; Mohr and Tebbe, 2006; Donovan et al., 2004; Reeson et al., 2003). In the two former techniques, bands may be further extracted from the gels and sequenced, enabling identification of specific populations.

These tools by which uncultured microorganisms are discovered and identified have become instrumental in microbial ecology. Yet, biochemical characterization and genetic manipulations are greatly improved when the target organism is isolated. Analysis of sequences originating from clone libraries or from DGGE/SSCP extracted bands can greatly facilitate the isolation of target bacteria. Identification directs the researcher toward specific bacterial groups and therefore toward adequate isolation protocols, thereby greatly increasing the odds of isolating or enriching for the target organism. Recent examples of this efficient strategy are the identification and isolation of *Asaia* sp. that form dominant populations in the Asian malarial mosquito vector *Anopheles stephensi* (Favia et al., 2007) and the enrichment for an aerobic phototrophic acidobacterium from a Yellowstone National Park spring (Bryant et al., 2007).

In our work on the medfly gut community, we make large use of a polyphasic approach that includes the isolation of culturable bacteria on growth media and molecular, culture independent analyses. The enormous majority of the medfly gut community is composed of various Enterobacteriaceae that were found using both approaches (see below). Further, direct and culture-based identification lead us to hypothesize that diazotrophic and pectinolytic functions are performed by many members of the gut community. Demonstration of these hypotheses was achieved using specific media and chemical analyses (Behar et al., 2005, and below). We further studied the relationship between gut bacteria, the insect's developmental stage and its fruit host, identified seasonal and geographical fluctuations in community structure, and isolated a minor but important component of the community (Behar et al., 2005, 2008a, 2008b, and see below).

Metagenomics can provide unequaled amounts of data on microbial community structure and function, especially when combined with large scale 16S rRNA gene library analyses. As an example, the hindgut microbiota of a wood termite was recently described using metagenomics (Warnecke et al., 2007). The diversity and richness of this bacterial community was revealed along with metabolic and enzymatic functions linked to it, such as CO₂ reductive acetogenesis, N₂ fixation, cellulose and xylan degradation genes, and lignocellulose degradation.

Novel sequencing equipment based on pyrosequencing, Illumina (Solexa), or SOLID technologies (Margulies et al., 2005; Metzker, 2005) add enormous power and very high throughput capacities to the researcher's tool box. First applications to the field of microbe–arthropod interactions have already led to tangible results: for example, the possible cause of colony collapse disorder (CCD) in *Apis mellifera*, the European honey bee, may be a dicistrovirus. This virus was rapidly identified by very large scale sequence analysis using pyrosequencing (Cox-Foster et al., 2007). Although this study was initiated to help identify the cause of the disease, it also provided much data on the composition of bacterial, fungal, and viral communities of the bee. It should be mentioned, however, that at present, the phylogenetic resolution of these high throughput approaches is rather limited due to the

short sequence reads. The greater precision achieved in this study was due to complementary analysis of 16S rRNA gene libraries.

New techniques have been proposed that expand the sensitivity of the PCR-based rRNA approach. The use of inosine at the 3' end of 16S rRNA-targeted primers instead of a specific base was shown to substantially increase the proportion of phyla that are poorly amplified, or not amplified at all, when universal primers are used (Ben-Dov et al., 2006). To date, this technique has only been applied to study a disease in corals (Barneah et al., 2007). We have experimented with suicide polymerase endonuclease restriction (SuPER) PCR, a novel rRNA-based approach (Green and Minz, 2005). Whereas inosine-based primers enable effective amplification of sequences missed by standard primers, in SuPER PCR, sequences yielding dominant amplicons can be selectively digested. This “frees” the reaction to amplify sequences originating in minor populations (Green and Minz, 2005). In our work, the application of SuPER PCR in a DGGE format yielded new banding patterns. Band analysis showed they all originated from various species of pseudomonads (more below).

Fruit flies and bacteria

The association between fruit flies and bacteria was first recognized in the beginning of the twentieth century, when, based on microscopic observations, Petri described symbiotic relations between the olive fly *Bactrocera oleae* and a microorganism. He suggested that this symbiont might be *Pseudomonas savastanoi*, a bacterial pathogen causing the olive knot disease (Petri, 1909).

Bacterial isolation from fruit flies and their oviposition sites were sporadically reported since 1934. However, beginning in the mid 1980s many studies were conducted on fruit fly–bacterial interactions (see reviews by Drew and Lloyd, 1987, 1991; Lauzon, 2003). These studies were based on traditional microbial methods such as viable plate counts and phenetic taxonomy and focused mainly on the bacteria inhabiting the digestive system of adult fruit flies (Table 11.1). Two species received quite a lot of attention—the Queensland fruit fly, *Bactrocera tryoni*, and the apple maggot fly, *Rhagoletis pomonella*. The bacteria found to be associated with *B. tryoni* were members of the family Enterobacteriaceae, mainly species of *Klebsiella* and *Enterobacter*, with *Klebsiella oxytoca* and *Enterobacter cloacae* as the most common species. These bacteria were also found in the different instars of the flies and in infested fruit (Fitt and O'Brien, 1985; Drew and Lloyd, 1987; Table 11.1).

Species of *Klebsiella* and *Enterobacter* are also commonly associated with *R. pomonella*. Lauzon described different associations, mainly with *Enterobacter agglomerans* and *Klebsiella pneumoniae* (Lauzon, 2003). Howard et al. (1985) found that *Klebsiella oxytoca* is the most common species associated with this fly throughout its life and suggested that this bacterium might be the equivalent symbiont in *R. pomonella* to *Pseudomonas savastanoi* in the olive fly (Howard et al., 1985; Table 11.1). However, the use of the rRNA approach has redefined the microbial community associated with the olive fly. Capuzzo et al. (2005) showed that the olive fly's symbiont is not *Pseudomonas savastanoi* (as suggested by Petri, 1909), and propose a novel bacterial species: *Erwinia dacicola*. A novel survey of 25 Tephritinae species revealed numerous new specific bacterial symbionts, all belonging to the Enterobacteriaceae (Mazzon et al., 2008).

Bacteria in the medfly

Until recently, the association between the Mediterranean fruit fly (medfly) and bacteria received little scrutiny. Marchini et al. (2002) studied the bacteria associated with the

Table 11.1 Bacteria Associated with Tephritid Fruit Flies

Tephritid Species	Source of Sample	Bacterial Species Found (most common species in bold)	Microbial Method	Reference
<i>Anastrepha ludens</i>	Adult gut	<i>Enterobacter cloacae</i> <i>Providencia spp.</i> , <i>Citrobacter koseri</i> <i>Enterobacter sakazakii</i> <i>Klebsiella pneumoniae</i> <i>Pseudomonas aeruginosa</i>	Classical	Kuzina et al. 2001
<i>A. ludens</i>	Adult crop + gut	<i>Citrobacter freundii</i> <i>Klebsiella oxytoca</i>	Classical	Martinez et al. 1994
<i>A. ludens</i>	Adult crop	<i>Enterobacter spp.</i> <i>Pseudomonas aeruginosa</i> <i>Pseudomonas spp.</i>	Classical	Martinez et al. 1994
<i>A. ludens</i>	Adult gut	<i>Klebsiella pneumoniae</i>	Classical	Martinez et al. 1994
<i>A. ludens</i>	Infested fruit	<i>Citrobacter freundii</i> <i>Klebsiella oxytoca</i>	Classical	Martinez et al. 1994
<i>Bactrocera cacuminata</i>	Adult gut, pupae, eggs, infested fruit	<i>Citrobacter freundii</i> <i>Klebsiella pneumoniae</i> <i>Pseudomonas spp.</i>	Classical	Fitt and O'Brien 1985
<i>B. cacuminata</i>	Host plant leaves and fruit surface	<i>Pantoea spp.</i> <i>Pantoea agglomerans</i>	Classical	Raghu et al. 2002
<i>B. jarvisi</i>	Adult gut, pupae, eggs, infested fruit	<i>Enterobacter agglomerans</i> <i>Enterobacter cloacae</i> <i>Enterobacter spp.</i> <i>Klebsiella pneumoniae</i> <i>Providencia spp.</i>	Classical	Fitt and O'Brien 1985
<i>B. jarvisi</i>	Adult gut, pupae, eggs, infested fruit	<i>Pseudomonas spp.</i>	Classical	Fitt and O'Brien 1985
<i>B. neohumeralis</i>	Adult gut, pupae, eggs, infested fruit	<i>Enterobacter cloacae</i> <i>Enterobacter spp.</i> <i>Pseudomonas spp.</i>	Classical	Fitt and O'Brien 1985
<i>B. oleae</i>	Adult esophageal bulb, gut, ovipositor	<i>Erwinia dacicola</i>	Molecular (16S rRNA gene)	Capuzzo et al. 2005
<i>B. oleae</i>	Adult esophageal bulb	<i>Pseudomonas savastani</i>	Classical	Petri 1909
<i>B. tryoni</i>	Adult crop, gut, mouthparts; host plant	<i>Enterobacter cloacae</i> <i>Klebsiella oxytoca</i> <i>Klebsiella ozaenae</i> <i>Pantoea agglomerans</i> <i>Providencia spp.</i>	Classical	Drew and Lloyd 1987

Continued.

Table 11.1 Bacteria Associated with Tephritid Fruit Flies (*Continued*)

Tephritid Species	Source of Sample	Bacterial Species Found (most common species in bold)	Microbial Method	Reference
<i>B. tryoni</i>	Adult gut, pupae, eggs, infested fruit	<i>Enterobacter agglomerans</i> <i>Enterobacter cloacae</i> <i>Klebsiella pneumoniae</i> <i>Providencia</i> spp. <i>Pseudomonas</i> spp.	Classical	Fitt and O'Brien 1985
<i>B. tryoni</i>	Adult crop, gut	<i>Klebsiella oxytoca</i> <i>Enterobacter cloacae</i>	Classical	Murphy et al. 1988; 1994
<i>Ceratitis capitata</i>	Adult gut	<i>Enterobacter</i> spp. <i>Klebsiella</i> spp.	Classical	Lauzon 2003
<i>C. capitata</i>	Adult esophageal bulb	<i>Enterobacter agglomerans</i> <i>Klebsiella oxytoca</i> <i>Enterobacter cloacae</i> <i>Pseudomonas putida</i> <i>Pseudomonas</i> spp.	Classical	Marchini et al. 2002
<i>C. capitata</i>	Adult gut, larvae, pupae, eggs, host plant	See Table 11.2	Molecular	Behar et al., 2005; 2008a; 2008; b
<i>Rhagoletis alternata</i>	Adult gut, larvae	<i>Enterobacter</i> spp. <i>Erwinia</i> spp.	Classical	Daser and Brandl 1992
<i>R. completa</i>	Adult esophageal bulb	<i>Klebsiella oxytoca</i> <i>Klebsiella ozaenae</i> <i>Klebsiella pneumoniae</i>	Classical	Howard et al. 1985
<i>R. cornivora</i>	Adult esophageal bulb	<i>Klebsiella oxytoca</i> <i>Klebsiella pneumoniae</i>	Classical	Howard et al. 1985
<i>R. electromorpha</i>	Adult esophageal bulb	<i>Klebsiella oxytoca</i> <i>Enterobacter cloacae</i> <i>Klebsiella ozaenae</i> <i>Klebsiella pneumoniae</i>	Classical	Howard et al. 1985
<i>R. mendax</i>	Adult esophageal bulb	<i>Klebsiella oxytoca</i> <i>Enterobacter agglomerans</i> <i>Enterobacter cloacae</i> <i>Klebsiella ozaenae</i> <i>Klebsiella pneumoniae</i>	Classical	Howard et al. 1985
<i>R. pomonella</i>	Adult esophageal bulb	<i>Klebsiella oxytoca</i> <i>Enterobacter agglomerans</i> <i>Enterobacter cloacae</i> <i>Klebsiella ozaenae</i> <i>Klebsiella pneumoniae</i> <i>Pseudomonas putida</i> <i>Pseudomonas</i> spp.	Classical	Howard et al. 1985

Table 11.1 Bacteria Associated with Tephritid Fruit Flies (Continued)

Tephritid Species	Source of Sample	Bacterial Species Found (most common species in bold)	Microbial Method	Reference
<i>R. pomonella</i>	Adult crop, gut, esophageal bulb	<i>Enterobacter agglomerans</i>	Classical	Lauzon et al. 1998; 2002
<i>R. pomonella</i>	Adult gut	<i>Klebsiella pneumoniae</i>	Classical	Lauzon et al. 1998; 2002
<i>R. pomonella</i>	Adult esophageal bulb	<i>Enterobacter cloacae</i>	Classical	Rossiter et al. 1983
<i>R. pomonella</i>	Adult esophageal bulb, eggs, larvae, pupae, infested fruit	<i>Klebsiella oxytoca</i>	Classical	Rossiter et al. 1983
<i>R. suavis</i>	Adult esophageal bulb	<i>Klebsiella oxytoca</i> <i>Enterobacter agglomerans</i> <i>Enterobacter cloacae</i> <i>Klebsiella ozaenae</i> <i>Klebsiella pneumoniae</i>	Classical	Howard et al. 1985
<i>R. tabellaria</i>	Adult esophageal bulb	<i>Klebsiella oxytoca</i> <i>Enterobacter agglomerans</i> <i>Enterobacter cloacae</i> <i>Klebsiella ozaenae</i> <i>Klebsiella pneumoniae</i>	Classical	Howard et al. 1985
<i>Tephritis conura</i>	Adult gut, larvae	<i>Erwinia</i> spp.	Classical	Daser and Brandl 1992
<i>Tephritis dilacerata</i>	Adult gut, larvae	<i>Enterobacter</i> spp.	Classical	Daser and Brandl 1992
<i>Urophora cuspidata</i>	Adult gut, larvae	<i>Erwinia</i> spp.	Classical	Daser and Brandl 1992
<i>Urophora solstitialis</i>	Adult gut, larvae	<i>Erwinia</i> spp.	Classical	Daser and Brandl 1992

esophageal bulb of the medfly using culture-dependent methods. They found that this organ harbors a discrete community of bacteria, comprised mainly, as in other fruit flies, of members of the family Enterobacteriaceae. Nevertheless, they also found some *Pseudomonas* spp., suggesting that the Enterobacteriaceae are not the sole community in the medfly's gut.

A systematic study of the structure and diversity of microbial communities in eggs, larvae, host fruit, pupae, and adult medflies, based on 16S rDNA sequences obtained from PCR-DGGE and from isolated colonies, revealed that members of the Enterobacteriaceae constitute the dominant populations in the medfly's gut (Table 11.2; Figure 11.1). Most prominent were species of *Klebsiella*, which were found in different combinations with

Table 11.2 Bacteria Isolated from the Medfly and Its Hosts

Sequence (Accession Numbers-NCBI)	Phylogenetic Identification	Source of Sample	Frequency Isolates/Samples
AY847180	<i>Pectobacterium cypripedi</i>	Adults	25/83
		Pooled eggs	4/4
		Larvae	9/12
		Pupae	7/8
DQ533879	<i>Enterobacter</i> spp.	Adults	2/83
		Larvae	5/12
		Pupae	3/8
DQ533880	<i>Enterobacter</i> spp.	Adults	14/83
DQ533881	<i>Enterobacter</i> spp.	Adults	40/83
		Pupae	3/8
DQ533882	<i>Enterobacter</i> spp.	Adults	8/83
		Rotting fruits	2/13
DQ533883	<i>Enterobacter gergoviae</i>	Adults	2/27
DQ533884	<i>Klebsiella oxytoca</i>	Adults	4/83
		Pupae	3/8
DQ533886	<i>Klebsiella oxytoca</i>	Adults	12/83
DQ533887	<i>Klebsiella oxytoca</i>	Adults	19/83
		Pupae	4/8
DQ533888	<i>Klebsiella oxytoca</i>	Adults	12/83
DQ533889	<i>Klebsiella oxytoca</i>	Adults	78/83
		Pooled eggs	1/4
		Rotting fruits	11/13
		Larvae	10/12
		Pupae	8/8
DQ533892	<i>Citrobacter freundii</i>	Adults	57/83
		Pooled eggs	1/4
		Rotting fruits	7/13
DQ533893	<i>Citrobacter freundii</i>	Adults	36/83
DQ533894	<i>Pectobacterium cypripedi</i>	Adults	7/83
		Pupae	2/8
DQ533896	<i>Providencia stuartii</i>	Adults	10/27
DQ533900	<i>Pseudomonas putida</i>	Adults	21/57
DQ533901	<i>Pseudomonas aeruginosa</i>	Adults	2/57
DQ533902	<i>Pseudomonas fluorescens</i>	Adults	20/57
DQ533904	<i>Pseudomonas</i> sp.	Adults	49/57
EF117864	<i>Enterobacter</i> spp.	Adults	7/83
		Larvae	6/12
		Pupae	2/8
EF645649	<i>Pantoea</i> spp.	Adults	4/83
		Pooled eggs	2/4
		Rotting fruits	2/13

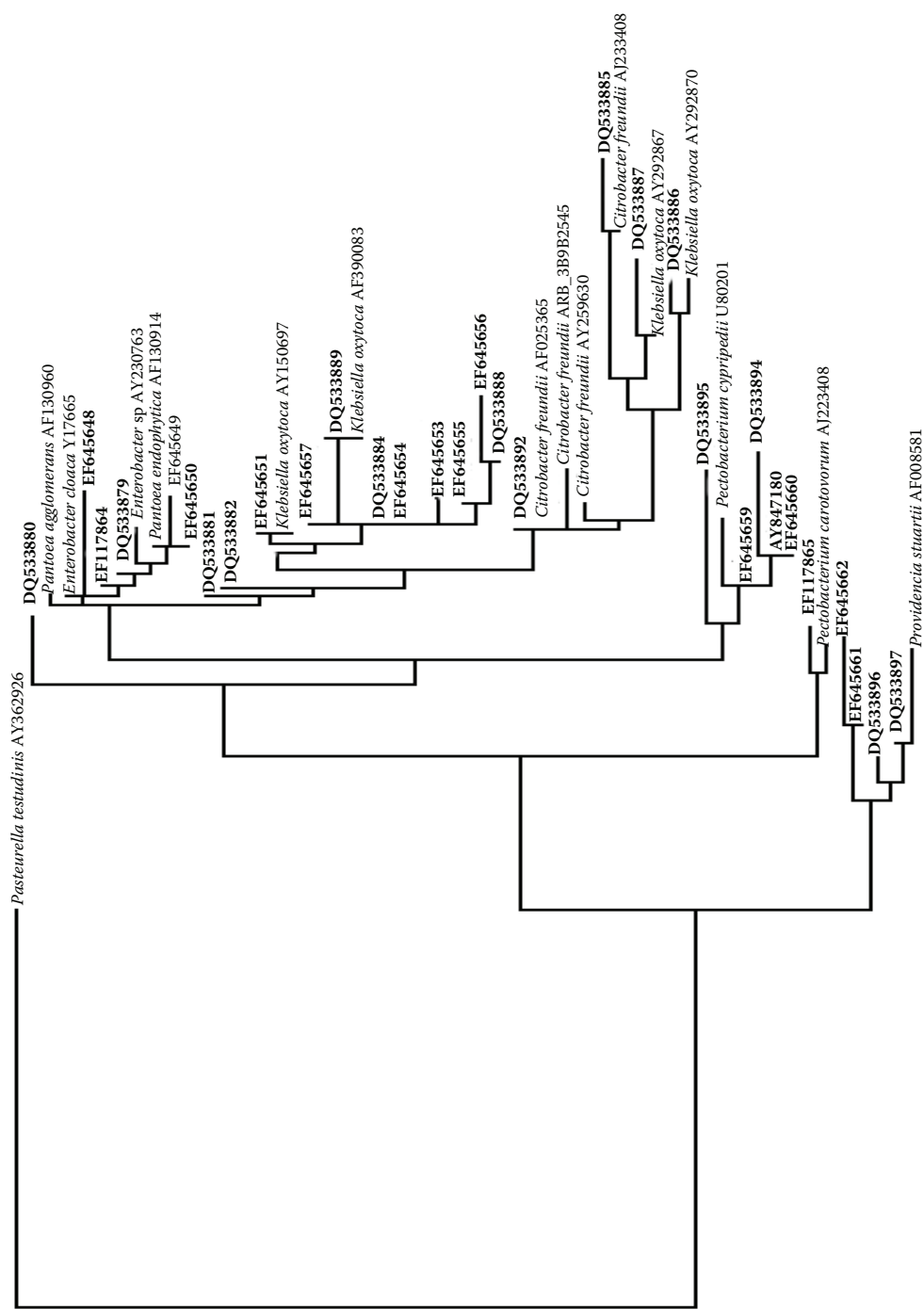


Figure 11.1 Phylogenetic tree of the Enterobacteriaceae community based on 16S rDNA sequence analysis of *Ceratitis capitata*-associated bacteria. The tree is based on maximum-likelihood analysis, using a 50% conservation filter. Parsimony analysis essentially yielded the same topology. Scale bar indicates 10% estimated sequence divergence. A homologous sequence from *Pasteurella testudinis* was used as an out group.

Citrobacter freundii, *Enterobacter* spp., *Pantoea* spp., *Pectobacterium* spp., and *Providencia stuartii* (Behar et al., 2005, 2008a).

These analyses also found that, as in other fruit flies, *Klebsiella oxytoca* is the most common species in the medfly's gut. Furthermore, a species of *Pectobacterium* was found to be commonly associated with the medfly, particularly in the larval stages (Table 11.2). Cluster analysis of the DGGE gels reveals that although this dominant community exhibits stable features, it also displays some plasticity, as the community's structure and diversity varied according to seasonal, ontogenetic, and geographical gradients (Behar et al., 2008a, 2008b).

When a novel technique called SuPER PCR (described above) was implemented on medfly gut samples analyzed by regular DGGE, and a minor, cryptic bacterial community was detected. Phylogenetic analysis identifies these bacteria as *Pseudomonas*, confirming that the Enterobacteriaceae do not form the sole community in the medfly gut (Table 11.2). Using selective media, *Pseudomonas*, which are known to be pathogenic to the nematode *C. elegans* and to *Drosophila* (Apidianakis et al., 2005; Hilbi et al., 2007) such as *P. aeruginosa*, were isolated (Table 11.2). The abundance of the cultivated *Pseudomonas* community was about 0.005% of the total bacterial population, confirming that this is indeed a minor population of several hundred bacteria per gut. However, as other *Pseudomonas* species detected by the SuPER PCR were not cultivated, the size of this community could be significantly higher.

PCR-DGGE analyses also supplied evidence that the enterobacterial community, composed mainly of *K. oxytoca*, *P. cypripedii*, *Pantoea* spp., and *Citrobacter freundii*, is present during all of the fly's developmental stages and that some elements are vertically transmitted from the female parent to its offspring during oviposition (Table 11.2; Behar et al., 2008a). Visual proof was achieved through the transformation of *E. agglomerans* and *Klebsiella pneumoniae* with fluorescent proteins and the subsequent ingestion of these bacteria by medflies (Lauzon et al., 2008). Upon examination, female medflies contained eggs that had a biofilm of the fluorescent bacteria that existed at the apical end of the eggs, the end that first enters host fruit tissue during oviposition. Scanning electron microscopy also showed a biofilm of bacteria at the apical end of the eggs (Lauzon et al., 2008). In addition, preliminary FISH analyses suggest that some Enterobacteriaceae may be located within the egg (Behar et al., unpublished). Strikingly, all these bacterial species are potential diazotrophs. *K. oxytoca*, *Pantoea* spp., and *P. cypripedii* are also known pectinolytic bacteria (Zinder and Dworkin, 2000). A newly eclosed adult fruit fly possesses a gut that is not fully developed and lacks numerous bacteria, typically observed in the gut of 18-hour-old adults (Lauzon and Potter, 2008). In nature, newly eclosed fruit flies generally remain relatively inactive during their first 24 hours of life. Perhaps this is to allow their gut to develop prior to ingestion of food or other noxious/toxic compounds, and for bacteria carried over from the pupal stage to grow and increase in number. Once a peritrophic membrane is formed that supports a biofilm of beneficial bacteria, the fly appears ready to begin to digest food and other compounds properly. The cycle of horizontal and vertical transmission begins again.

The vertical transmission and ubiquity throughout the life cycle of the medfly's gut enterobacterial community of pectinolytic and diazotrophic bacteria suggests that these bacteria may have a significant contribution to some components of their host's fitness. In addition, as most of these Enterobacteriaceae are also associated with other tephritid species (Table 11.1), and many are contained within specific organs, it appears that these bacteria have a long standing evolutionary relationship with their hosts, and that these fly-bacteria interactions are of a symbiotic nature. The importance of this enterobacterial

community to the medfly, and perhaps to all tephritids, can be determined by examining the possible functions these bacteria may perform in relation to their host's biology.

Possible functions of the bacterial community

Nitrogen fixation and cycling

Obtaining sufficient nitrogen is a major challenge for insects utilizing diets with high carbon:nitrogen ratios. Termites were studied in this context and are known to compensate for their skewed dietary C:N balance by nurturing a diazotrophic (nitrogen fixing) gut microbiota and acquiring much of their nitrogen directly from the atmosphere (Breznak et al., 1973; Breznak, 1982). A number of reports suggest that nitrogen provisioning bacteria may be more ubiquitous among arthropods than previously suspected (reviewed by Nardi et al., 2002; see also Kneip et al., 2007).

Many of the bacteria resident in the digestive system of fruit flies, notably species of *Klebsiella* (*K. oxytoca* and *K. pneumoniae*) and *Enterobacter* (*E. agglomerans* and *E. cloacae*), are diazotrophic. As the diet of flies is poor in protein through all stages of development (Yuval and Hendrichs, 2000; Yuval et al., 1998), several attempts have been made to test the hypothesis that these enterobacteria contribute to the nutritional ecology of these flies by fixing atmospheric nitrogen while resident in the fly gut. Howard and coworkers (1985) found no evidence in favor of nitrogenase activity within larvae and adults of a laboratory strain of *R. pomonella*. However, nitrogen fixation was detected in *B. tryoni* after the flies were allowed to feed on cultured *Klebsiella* and *Enterobacter* bacteria (Murphy et al., 1988). Recently, we demonstrated that nitrogen fixation occurs *in vivo* in wild medflies and results from the activity of stable and dominant populations of diazotrophic Enterobacteriaceae located in the gut of these flies (Behar et al., 2005). Nitrogen fixation was estimated to proceed at a rate that could provide as much as 6 µg protein/fly/day (Behar et al., 2005)—a significant proportion of the medfly's daily nitrogen requirement (Galun et al., 1985). Furthermore, nitrogen fixation may also be taking place within medfly larvae. Behar et al. (2008a) found that a gene responsible for fixing atmospheric nitrogen (*nifH*) is expressed within the larval gut. As larvae experience a high C:N environment within the fruit and protein is essential for larval growth (Yuval and Hendrichs, 2000, Kaspi et al., 2002), nitrogen fixation could contribute to larval nitrogen diet by supplying necessary nitrogenous compounds. As most of these diazotrophic Enterobacteriaceae also constitute the dominant intestinal populations of several other tephritids (Table 11.1), nitrogen fixation may be more common among tephritid fruit flies than previously accounted for.

Bacteria may further contribute to the nitrogen budget of their hosts by recycling nitrogenous waste products (namely uric acid and ammonia) back into usable compounds. Termites as well as cockroaches utilize the uricolytic capabilities of their associated symbionts in order to conserve nitrogen (Potrikus and Breznak 1980b, 1980a, 1981; Cochran, 1985). It has been suggested that some enterobacterial populations (e.g., *Enterobacter* spp.) produce uricase, an enzyme degrading uric acid into allantoin, which is later degraded into urea (as shown in *Rhagoletis pomonella* by Lauzon et al., 2000), and urease, an enzyme degrading urea into ammonia (e.g., by *Providencia stuartii*, *K. oxytoca*, and *E. gergoviae*; Zinder and Dworkin, 2000).

Bird feces, a major source of protein for fruit flies, also serve as a major reservoir of bacteria, such as *Enterobacter* spp. Prokopy and coworkers (1993) demonstrated that attraction to bird feces ceases with the addition of antibiotics. Therefore, it may be that fruit flies

horizontally acquire bacteria to degrade uric acid in the adult fly midgut to a usable form (Lauzon et al., 2000).

The sensor–receptor complex regulating ammonia levels in bacterial cells may constitute a component of the regulatory process of the nitrogen fixation regulatory proteins in *Klebsiella pneumoniae* (Zhang et al., 2001). This might suggest that the combination of Enterobacteriaceae with different enzymes and sensors involved in nitrogen fixation/uric-acid cycle in the fly’s gut may greatly facilitate rapid adaptive responses to fluctuating levels of nitrogen availability and couple the fly’s nitrogen metabolism with bacterial activities.

As adults, medflies and other fruit flies are anautogenous and need to acquire protein in order to fulfill their reproductive potential (Drew and Yuval, 2000). A steady supply of fixed and/or recycled nitrogen, generated by internal symbionts, could provide the protein needed to facilitate egg production in females and high sexual activity in males, particularly under poor dietary conditions. Flies could benefit directly from such activity, by assimilating the ammonia generated in these processes and using it for amino acid synthesis, or indirectly by simply digesting their gut bacteria. The exact contribution of intestinal bacteria to the nitrogen budget of the flies still awaits clarification.

Carbon metabolism

Medfly larvae are known to produce several proteolytic enzymes as well as carbohydrases during their development (Silva et al., 2006) and hence do not seem to depend on bacteria for the digestion of protein and simple sugars—as suggested for other fruit flies (Hagen, 1966; see also Fitt and O’Brien, 1985). However, their ability to degrade polysaccharides seems to be limited (see Silva et al., 2006). Bacteria, on the other hand, are excellent at degrading polysaccharides such as cellulose and pectin—an attribute that was previously shown to accompany larval development in *Rhagoletis pomonella* (Rossiter et al., 1983).

Pectinolytic Enterobacteriaceae, mainly *K. oxytoca* and *Pectobacterium* spp., were indeed found to comprise the dominant gut bacterial populations during the larval stages of the medfly (Behar et al., 2008a). Because these larvae need to rapidly acquire sufficient nutrients in order to graduate to the next ontogenetic stage (Kaspi et al., 2002), bacteria-assisted pectinolysis within the fruit may contribute to the larval carbon diet by providing an additional carbon source of readily metabolizable sugars for the growing larvae. Additionally, by macerating the fruit cell walls, pectinolysis may also provide the larvae a more habitable environment, and assist in movement and emergence from the fruit. As viscosity or frictional forces decrease in a rotting fruit, so would the energetic cost of movements. Very few studies have addressed this subject linked to biomechanics (e.g., Podolsky, 1994). Furthermore, the ample supply of readily metabolizable carbohydrates produced by pectin degradation may also fuel the energy-demanding nitrogen-fixation process presumed to occur within the larvae.

Adult flies, on the other hand, obtain readily available sugars from fruit juices, honeydew, and nectar (Tsitsipis, 1989; Drew and Yuval, 2000) and therefore may not need a large pectinolytic microbiota. The observed decline in the titer of pectinolytic bacteria in the gut during the adult stage (Behar et al., 2008a) is consistent with this claim. More studies are needed to critically examine the contribution of the pectinolytic enterobacterial community to larval development.

Communication

Foraging fruit flies have long been known to be attracted to volatiles originating from bacterial catabolism of substrates containing protein (reviewed by Drew and Lloyd, 1991; Lauzon, 2003). Although ammonia seems to be a universal attractant, other volatiles of bacterial origin have been shown to attract fruit flies (Drew and Faye, 1988; Robacker and Flath, 1995; Robacker and Bartelt, 1997; Robacker and Lauzon, 2002; Epsky et al., 1998). Such behavior probably represents an adaptation for locating protein sources in the field, and seems not to be directed exclusively to bacteria associated with fruit flies (Robacker et al., 1998; Lauzon, 2003). Thus, bacteria contribute to the chemical ecology of pest tephritids by affecting their spatial distribution and highlighting resource rich spots.

Another aspect of fruit fly bacteria interactions involves volatiles of bacterial origin that serve as semiochemicals affecting adult behavior. Some fruit flies are attracted to bacteria isolated from oviposition wounds and held free of a medium (MacCollom et al., 1992, 1994). Accordingly, volatiles produced by bacteria may represent more than just a promise for a protein meal, and mediate more complex behaviors affecting fitness. That bacteria or their metabolites act in communication is well documented from a variety of insects: hindgut bacteria were shown to enhance social interactions by contributing to pheromone synthesis in the desert locust, *Schistocerca gregaria* (*Enterobacter*, *Pantoea*, *Klebsiella* spp.; Dillon et al., 2000, 2002), and are suspected to do so also in the cockroach, *Periplaneta americana* (Cruden and Markovetz, 1987). Aggregated oviposition was demonstrated to depend on olfactory cues derived from bacteria deposited with the eggs in onion maggot flies (*Delia antiqua*) (Judd and Borden, 1992). In this case *Pectobacterium carotovorum* may be the bacterium involved (see Judd and Burden, 1992, and references therein). Similar behavior was also recorded in the house fly, *Musca domestica*, and was attributed to the proliferation of maternally derived *Klebsiella oxytoca* bacteria on the eggs and oviposition substrate (Lam et al., 2007). Despite the use of host marking pheromones that deter other females from ovipositing in the same fruit, medfly females are also known to occasionally oviposit in an aggregated fashion (Díaz-Fleischer et al., 2000). Aggregated oviposition may be in the best interest of females, especially when ovipositing into large fruits, because the crowded development of larvae can strongly inhibit the occurrence of pathogenic agents in rotting, decomposing substrates (e.g., Rohlf et al., 2005). However, overcrowding or unsynchronized egg hatch could result in strong competition among the larvae. Bacteria deposited with the eggs into the host may produce volatile cues that provide arriving gravid females with information on the density and age of eggs already incubating within the fruit. Such information, along with pheromonal cues, could allow females to make optimal reproductive decisions.

The intestinal microbiota may also affect the fitness of male medflies. Ben-Yosef and coworkers (2008) recently examined the effects of intestinal bacteria on medfly fitness and its relation to diet. A significant reduction in sexual competitiveness of males fed with antibiotics while provided with all nutritional requirements was evident. On the other hand, clearing the gut of bacteria did not affect the ability of males fed only on sugar to achieve copulations. These findings are compatible with the work of Niyazi et al. (2004) who demonstrated a mating advantage in probiotically treated sterile males fed with protein. One way to explain these results is that intestinal bacteria influence male copulatory success by qualitatively or quantitatively contributing to pheromone production. Protein may be a prerequisite for such activity due to its positive effects on pheromone emission and copulatory success (Papadopoulos et al., 1998; Blay and Yuval, 1997). By synthesizing pheromonal precursors or alternatively modifying existing molecules produced by the

males, hindgut bacteria may provide the females with information about the male's health and nutritional status. These may be important parameters influencing mate choice in Lek mating systems, such as that of the medfly (Field et al., 2002), where males provide the females with nothing but their genes.

Defense against pathogens

Relatively little information exists in the area of tephritid pathology even though fruit flies consume and are exposed to insect pathogens such as cricket paralysis virus (Manousis and Moore, 1987), reoviruses and reo-like viruses (Plus et al., 1981a, 1981b; Lauzon, unpublished), *Wolbachia* (Kittayapong et al., 2000; Riegler and Stauffer, 2002; Selivon et al., 2002; Zabalou et al., 2004; Rocha et al., 2005), microsporidia (Fujii and Tamashiro, 1972), and *Serratia marcescens* (Steinhaus, 1959; Grimont and Grimont, 1978; Lauzon et al., 2002). Isolates of the latter were lethal to *Rhagoletis pomonella* (Lauzon et al., 2003).

Moreover, as mentioned earlier, some of the *Pseudomonas* strains forming the minor yet common and stable community in the medfly's gut are pathogenic. When higher than natural levels of *Pseudomonas aeruginosa* were orally introduced to the medfly's digestive system they reduced host longevity, while ingesting higher levels of the medfly's gut enterobacterial community improved host longevity (Behar, unpublished). These results suggest that at least part of the *Pseudomonas* community present in the gut cause damage to its medfly host when occurring at, or reaching, high densities.

The gut microbiota of silkworm larvae and locusts, among them the enterobacterium *Pantoea agglomerans*, provide a buffering action to help prevent the proliferation of pathogens (reviewed by Dillon and Dillon, 2004). Because the medfly's gut enterobacterial community dominantly establishes during the adult stage within the medfly's gut (Behar et al., 2008a) and contributes to their host longevity (as mentioned above), we postulate that by preventing the establishment and proliferation of harmful bacteria, the Enterobacteriaceae community may play a similar role in the medfly's gut. Thus, by keeping the *Pseudomonas* community in check, the dominant establishment of the Enterobacteriaceae community within the medfly's gut contributes to the fly's longevity, acting as a physical barrier against deleterious (foreign and indigenous) bacteria. More information needs to be acquired on pathogens because mass rearing programs must include plans to control and/or eliminate these microorganisms within the rearing facility. During studies that showed that a diet for medflies that includes beneficial symbionts improved the gut of irradiated flies used in the sterile insect technique (Lauzon and Potter, 2008) and their mating performance (Niyazi et al., 2004), Lauzon also found that bacteria that typically resided in the facility diet were eliminated or did not become established when the beneficial symbionts were present (unpublished). This dynamic decreases the need for antimicrobial use in mass rearing and may reflect a protective mechanism exerted by beneficial symbionts for fruit flies.

Phytopathology

Larval development within the fruit is accompanied by a rapid deterioration of the fruit pulp. During oviposition, fly-associated Enterobacteriaceae, mainly *Citrobacter freundii*, *Klebsiella oxytoca*, *Pantoea* spp., and *Pectobacterium cypripedii* (Table 11.2), are transmitted to the fruit along with the eggs and subsequently proliferate within it. Combined with feeding activity of larvae, these bacteria accelerate fruit decay (Behar et al., 2008a). Some species of the enterobacterial community in the medfly's gut, such as *Pantoea* spp. and *Pectobacterium cypripedii*, are known phytopathogens due to their ability to degrade pectin

(Zinder and Dworkin, 2000). Strains of *K. oxytoca* and *Pectobacterium* spp. isolated from the medfly's gut caused decay in potatoes (Behar, unpublished)—a known test for pectinolytic activity by bacteria (Page et al., 2001). In this capacity medflies (and other tephritids) may act as vectors of phytopathogenic bacteria. Because more than 300 species of fruit are confirmed as hosts for ovipositing medfly females, this mechanism may have a major agricultural significance.

Interactions at the ecosystem level

The host plant has been identified as a mediator between fruit flies and bacteria (Drew and Lloyd, 1987). This concept can be extended by looking at the fruit within which larvae develop as a “microbial hub.” Under natural conditions, oviposition hosts can be shared by conspecifics and, although the medfly usually is the first to attack the fruit and precipitate its decay, by other species. In nature, these other species are often other flies (mainly *Drosophilids*), beetles, and the attendant community of natural enemies, who rapidly proliferate within the decaying fruit. A number of interesting questions arise: what brings about or inhibits sharing of resources (the decaying fruit) with other species? Is there a gain of fitness, and are microorganisms involved in this gain (Rohlf and Hoffmeister, 2003)? Natural bacterial populations of *Drosophila* may be quite different from those of the medfly (Cox and Gilmore, 2007; Corby-Harris et al., 2007). If this is true for populations sharing larval feeding sites, how is this selectivity achieved, and can members of the community of one insect species colonize the other? Finally, the intriguing possibility of lateral gene transfer occurring in this setting arises. Such a process could contribute to the evolution of the interactions between the insects and their microbial partners, as well as to the diversity and fitness of the bacterial populations involved.

Conclusions

The associated bacterial community of the Mediterranean fruit fly has the potential to perform different functions during the fly's life cycle that correspond to the specific needs of each developmental stage. This community affects the fly's physiology and chemical ecology, and possibly mediates interspecific interactions at the community level. This perspective is summarized in Figure 11.2.

Our work has shown that the medfly supports a large bacterial community mainly composed of enterobacterial populations of limited diversity at the genus level. Vertical transmission of at least part of this microbial community has been demonstrated. However, what fraction of this community and the precise mechanism(s) by which it is transmitted are still not fully elucidated. It should also be emphasized that other microbes such as fungi (filamentous and yeasts) and protozoa may also inhabit the digestive tract and be present in the rotting fruit. Nothing is known on this subject. Nevertheless, we suggest that larvae begin their lives equipped with an “enterobacterial package” of pectinolytic and diazotrophic bacteria of maternal origin, which are transmitted together with the eggs into the fruit during oviposition. These bacteria subsequently establish and proliferate within the larvae as well as in the fruit, and provide the larvae with ample carbohydrates as well as protein due to pectin degradation and possibly nitrogen fixation. During the adult stage this enterobacterial community continues to establish within the fly's gut, contributing to its longevity perhaps by acting as the first line of defense against deleterious bacteria. At this stage, nitrogen fixation performed within the gut, coupled with recycling of nitrogenous metabolic waste, may provide the adults with the nitrogen-based building blocks

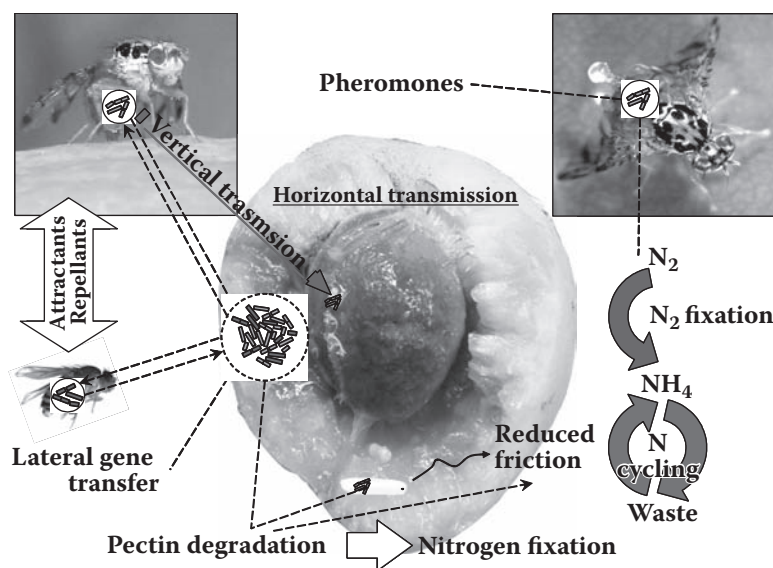


Figure 11.2 (Color figure follows p. 238.) Bacterial functions in the medfly life cycle. This depiction shows known as well as possible interactions occurring between ovipositing female medflies (upper left), male medflies (upper right), medfly larvae (in the fruit), members of their associated bacterial community (green rods), other conspecific and heterospecific insects (lower left) and their own associated bacterial communities (red rods), and the fruit. Color legend for the mentioned functions, interactions, or mechanisms: green, demonstrated; purple, circumstantial evidence; blue, hypothetical (see main text for details).

needed to fuel reproduction. These metabolic functions may form an integral part of the nutritional ecology of medflies, especially under poor nutritional conditions. Within their fly host, bacteria enjoy a protected environment, abundance of nutrients, and can also use the fly as a vector for distribution.

Understanding the association between the medfly and its microbiota is of major agricultural significance as it can be applied to improve the sterile male technique or in novel biocontrol strategies aimed at destabilizing some of the important functions performed by the microflora of wild flies.

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Feminizing *Wolbachia* and the evolution of sex determination in isopods*

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Introduction

Isopods, along with their relatives the amphipods, are the only crustaceans of which strictly terrestrial species exist. About half of the 8,000 isopod species described are terrestrial (Schmalfuss, 2003), and they can be used as potential guides to ecosystem activity in cultivated grasslands (Souty-Grosset et al., 2005). Terrestrial isopods (or woodlice) have been known for a long time to display aberrations in sex ratio both in the wild and in laboratory lineages (Vandel, 1941). One of the most intensively studied examples, the pill bug, *Armadillidium vulgare*, has long been known to harbor intracellular microorganisms (Martin et al., 1973) responsible for inducing the development of genetic males into functional females (Legrand and Juchault, 1970). The phylogenetic status of these bacteria remained

* This chapter is dedicated to Pierre Juchault, for his pioneering and influential work on sex determination in crustaceans.

unknown for almost two decades following their discovery, until they were identified as *Wolbachia pipientis*, on the basis of morphological and life cycle features (Rigaud et al., 1991). This taxonomic assignment was formally confirmed one year later by sequencing of the 16S and 23S ribosomal RNA genes (Rousset et al., 1992). In this review, we highlight recent results that have offered new insight into our understanding of the *Wolbachia*/crustacean endosymbiosis, both at functional and at evolutionary levels.

Wolbachia diversity

The Wolbachia pandemic in crustaceans

The presence of intracellular microorganisms in isopod crustaceans has been suspected for quite some time (reviewed in Rigaud, 1997). However, their formal characterization and study have been hampered by the impossibility of culturing them. In this respect, the development of PCR and sequencing in the early 1990s represented crucial technical advances for endosymbiont taxonomic assignment and research. Hence, *Wolbachia* molecular identification was first confirmed in two isopods, *A. vulgare* and *Porcellio dilatatus petiti*, as early as 1992 (Rousset et al., 1992). By the turn of the millennium, *Wolbachia* presence had been reported in 22 isopod species (Bouchon et al., 1998). Overall, *Wolbachia* prevalence was estimated to ~35% of isopod species, and ~46% of terrestrial isopods only (Bouchon et al., 1998). Interestingly, we have recently detected *Wolbachia* presence in six terrestrial isopod species that had tested negative in the previous screening by Bouchon et al. (1998). This allows us to reevaluate *Wolbachia* prevalence in isopods to ~47%. If only terrestrial isopods are considered, *Wolbachia* prevalence reaches ~61%. In excellent agreement with this estimate, ~62% of terrestrial isopod species tested were found to harbor *Wolbachia* in another screening of species from Tunisia (Ben Afia Hatira et al., 2008). Overall, *Wolbachia* infection is confirmed in 39 isopod species at the time of this writing (Table 12.1). Therefore, *Wolbachia* are highly prevalent in isopod crustaceans.

Is *Wolbachia* infection restricted to isopods? Extensive surveys in five different crustacean orders (Amphipoda, Isopoda, Tanaidacea, Cumacea, and Decapoda) initially suggested that *Wolbachia* infection was restricted to isopods (Bouchon et al., 1998). This view changed, however, when *Wolbachia* infection was reported in two amphipod species (Cordaux et al., 2001). Isopods and amphipods represent two distinct but closely related orders within the superorder Peracarida (class Malacostraca) (Martin and Davis, 2001). In fact, *Wolbachia* infection among amphipods is probably more widespread because we have recently detected *Wolbachia* in a third amphipod species (unpublished results). Furthermore, evidence is accumulating that *Wolbachia* bacteria infect a wide range of crustacean species, because they have recently been discovered in two additional crustacean classes: Ostracoda (Baltanas et al., 2007) and Maxillopoda (unpublished results). These results are significant because the three classes in which *Wolbachia* infection has been reported to date (Malacostraca: ~22,000 species; Ostracoda: ~13,000 species; Maxillopoda: ~15,000 species) encompass ~96% of all described crustacean species (Martin and Davis, 2001). In sum, our current understanding of the extent of *Wolbachia* infection among crustaceans (Table 12.1) is probably only the tip of the iceberg, but the emerging pattern is that *Wolbachia* infection is probably widespread among crustaceans in general, as it is in insects (Rigaud, 1999).

Table 12.1 List of Crustacean Species (Classification after Martin and Davis 2001) Infected by Wolbachia Bacteria, as of February 2008

Class	Subclass (SC) or Superorder (SO)	Infraclass (I) or Order (O)	Suborder (S) or Family (F)	Species	Phenotype ^a	Reference for Molecular Identification
Maxillopoda	Theostraca (SC)	Cirripedia (I)	Lepadomorpha (S)	<i>Lepas anatifera</i>	?	Unpublished observations
	Copepoda (SC)	Cyclopoida (O)	Cyclopidae (F)	<i>Cyclops abyssorum</i>	?	Unpublished observations
	Podocopa (SC)	Podocopida (O)	Cyprididae (F)	<i>Herpetocypris brevicaudata</i>	?	Baltanas et al. (2007)
Ostracoda				<i>Eucypris virens</i>	?	Baltanas et al. (2007)
				<i>Orchestia gammarellus</i>	?	Cordaux et al. (2001)
				<i>Talorchestia deshayesii</i>	?	Cordaux et al. (2001)
Malacostraca	Peracarida (SO)	Amphipoda (O)	Gammaridea (S)	<i>Talitrus saltator</i>	?	Unpublished observations
				<i>Sphaeroma hookeri</i>	F?	Bouchon et al. (1998)
				<i>Sphaeroma rugicauda</i>	F?	Bouchon et al. (1998)
		Isopoda (O)	Flabellifera (S)	<i>Asellus aquaticus</i>	F?	Bouchon et al. (1998)
				<i>Helleria brevicornis</i>	?	Bouchon et al. (1998)
				<i>Ligia oceanica</i>	F?	Bouchon et al. (1998)
			Aselotta (S)	<i>Haplophthalmus danicus</i>	?	Bouchon et al. (1998)
				<i>Oniscus asellus</i>	F	Bouchon et al. (1998)
				<i>Oniscus lusitanus</i>	?	Bouchon et al. (1998)
			Oniscidea (S)	<i>Chaetophiloscia elongata</i>	F?	Bouchon et al. (1998)
				<i>Philoscia muscorum</i>	F?	Bouchon et al. (1998)
				<i>Platyarthrus hoffmannseggii</i>	?	Unpublished observations
				<i>Cubaris murina</i>	?	Unpublished observations
				<i>Armadillidium album</i>	F?	Bouchon et al. (1998)
				<i>Armadillidium granulatum</i>	?	Ben Afia Hatira et al. 2008)

Table 12.1 List of Crustacean Species (Classification after Martin and Davis 2001) Infected by *Wolbachia* Bacteria, as of February 2008 (Continued)

Class	Subclass (SC) or Superorder (SO)	Infraclass (I) or Order (O)	Suborder (S) or Family (F)	Species	Phenotype ^a	Reference for Molecular Identification
				<i>Armadillidium sulcatum</i>	?	Ben Afia Hatira et al. (2008)
				<i>Armadillidium tunisiense</i>	?	Ben Afia Hatira et al. (2008)
				<i>Armadillidium vulgare</i>	F	Cordaux et al. (2004); Rousset et al. (1992)
				<i>Schizidium tiberanum</i>	?	Bouchon et al. (1998)
				<i>Cylisticus convexus</i>	CI	Bouchon et al. (1998)
				<i>Leptotricus panzerii</i>	?	Ben Afia Hatira et al. (2008)
				<i>Porcellio albinus</i>	?	Ben Afia Hatira et al. (2008)
				<i>Porcellio buddelundi</i>	?	Ben Afia Hatira et al. (2008)
				<i>Porcellio dilatatus dilatatus</i>	?	Unpublished observations
				<i>Porcellio dilatatus petiti</i>	CI	Rousset et al. (1992)
				<i>Porcellio dispar</i>	?	Bouchon et al. (1998)
				<i>Porcellio djahizi</i>	?	Ben Afia Hatira et al. (2008)
				<i>Porcellio laevis</i>	?	Ben Afia Hatira et al. (2008)
				<i>Porcellio lamellatus</i>	?	Ben Afia Hatira et al. (2008)
				<i>Porcellio scaber</i>	F?	Bouchon et al. 1998)
				<i>Porcellio spinicornis</i>	?	Bouchon et al. (1998)
				<i>Porcellio variabilis</i>	?	Bouchon et al. (1998)
				<i>Porcellionides cingendus</i>	?	Unpublished observations
				<i>Porcellionides pruinosus</i>	F?	Marcadé et al. (1999); Michel-Salzat et al. (2001)
				<i>Porcellionides sexfaciatus</i>	?	Ben Afia Hatira et al. (1998)
				<i>Proporcellio quadriseriatus</i>	?	Bouchon et al. (1998)
				<i>Hemilepistus reaumuri</i>	?	Unpublished observations
				<i>Trachelipus rathkei</i>	?	Unpublished observations

Phylogenetic relationships among Wolbachia strains

Wolbachia bacteria belong to the order Rickettsiales, within the alpha subdivision of the Proteobacteria, and they are closely related to *Ehrlichia* and *Anaplasma* bacteria (O'Neill et al., 1992; Rousset et al., 1992). Molecular studies have demonstrated that *Wolbachia* genetic diversity can be subdivided into at least 8 clades of related strains, termed supergroups A to H (Lo et al., 2007; Werren et al., 1995). Phylogenetic analyses based on various *Wolbachia* markers (16S rRNA, *ftsZ*, *wsp*, and *groE*) have consistently indicated that all isopod *Wolbachia* strains characterized to date belong to the *Wolbachia* B-supergroup (Bouchon et al., 1998; Cordaux et al., 2001; Verne et al., 2007). With the exception of one *Wolbachia* strain isolated from *Porcellionides pruinosus*, all other strains from terrestrial isopods form a monophyletic group of strains (Bouchon et al., 1998; Cordaux et al., 2001) related to those of the mosquitoes *Culex pipiens* and *Aedes albopictus* (Cordaux et al., 2001). Interestingly, *Wolbachia* strains from nonterrestrial isopods, amphipods, and maxillopods are closely related to those of terrestrial isopods. Therefore, crustacean *Wolbachia* strains globally appear to cluster in two distinct groups within the *Wolbachia* B-supergroup (Cordaux et al., 2001). The only exception to this pattern to date is found in the ostracod *Eucypris virens*, which may harbor an A-supergroup *Wolbachia* strain (Baltanas et al., 2007). However, no sequence data and phylogenetic analysis was presented in the study; additional analyses are therefore warranted before this result can be confirmed.

Comparison of the phylogenetic relationships of crustacean hosts and their respective *Wolbachia* strains show limited consistency (Bouchon et al., 1998; Cordaux et al., 2001; Michel-Salzat and Bouchon, 2000). This observation suggests that the evolutionary dynamics of crustacean *Wolbachia* strains involves horizontal transmission of symbionts among hosts (Bouchon et al., 1998; Cordaux et al., 2001). The occurrence of horizontal transfers of *Wolbachia* between unrelated hosts is now a well-accepted concept (Vavre et al., 1999; Werren et al., 1995), but the mechanisms mediating such transfers are still poorly understood.

It has been known for a long time that horizontal transfers of *Wolbachia* in isopods have been successfully obtained by microinjection (e.g., Bouchon et al., 1998; Juchault et al., 1994). Natural routes for horizontal transmission have also been investigated in isopods based on the initial observation that *Wolbachia* bacteria are found within hemocytes of *A. vulgare* (Rigaud et al., 1991). Indeed, it was elegantly demonstrated that *Wolbachia* transfers may occur between the hemolymph of infected and noninfected isopods (Rigaud and Juchault, 1995). This is further supported by the fact that terrestrial isopods are gregarious and that ~10% of animals are typically injured in natural populations (Rigaud and Juchault, 1995). We have also investigated the possibility of natural horizontal transmission between isopods and ecologically associated species such as predators and parasites, using phylogenetic methods (Cordaux et al., 2001). We found that parasitoid flies and phoretic mites of isopods harbor *Wolbachia* strains that are closely related to those of isopods, thus providing indirect evidence for two potential routes for horizontal transfers involving crustaceans (Cordaux et al., 2001).

Wolbachia diversity within species and individuals

The extent of *Wolbachia* diversity within species has been investigated in two terrestrial isopods: *P. pruinosus* (Marcadé et al., 1999; Michel-Salzat et al., 2001) and *A. vulgare* (Cordaux et al., 2004; Verne et al., 2007). Three distinct *Wolbachia* strains have been identified within each isopod species. In *P. pruinosus*, two strains (*wPruI* and *wPruII*) exhibit ~5% nucleotide divergence based on the variable *wsp* gene (Michel-Salzat et al., 2001). In comparison, the

third strain (*wPruIII*) shows ~20% nucleotide divergence with *wPruI* and *wPruII* based on the *wsp* gene. Interestingly, two types of *Wolbachia* infections are found in the *P. pruinus* complex of species (Lefebvre and Marcadé, 2005): (1) populations with *Wolbachia* in both males and females, with a prevalence of ~90%, and (2) populations with *Wolbachia* only in females, with a prevalence of ~60% (Marcadé et al., 1999). We observed that there is no strong association between *Wolbachia* strain distribution and infection patterns. Indeed, the two most closely related strains *wPruI* and *wPruII* were found associated with different infection patterns, whereas the two distantly related strains *wPruII* and *wPruIII* were found to exhibit the same infection pattern (Michel-Salzat et al., 2001). These results suggest that host genetic backgrounds may play an important role in the expression of *Wolbachia* phenotypes in *P. pruinus*.

In *A. vulgare*, two strains (*wVulC* and *wVulM*) exhibiting ~5% nucleotide divergence based on the *wsp* gene have been shown to induce feminization of genetic males (Cordaux et al., 2004). The third strain (*wVulP*) is essentially identical to *wVulC*, except for a ~60 bp-long portion of the *wsp* gene that is more closely related to *wVulM*, suggesting that *wVulP* is a recombinant strain between *wVulC* and *wVulM* (Verne et al., 2007). The phenotype induced by *wVulP* on its hosts is currently unknown, but if it has any effect, it is likely to be a feminizing strain, based on sequence similarity to *wVulC*. The discovery of the recombinant *wVulP* strain has interesting evolutionary implications because it implies that two feminizing *Wolbachia* strains (*wVulC* and *wVulM*) have coexisted at some point within the same cytoplasm. This is a quite unexpected result because no crustacean individual has been found to harbor multiple *Wolbachia* strains to date, although multiple strains can coexist within populations (Verne et al., 2007). Thus, infections of single individuals with multiple feminizing *Wolbachia* strains seem unstable in natural populations, in agreement with theoretical predictions (Caubet et al. 2000). Overall, these results suggest that the occurrence of multiple *Wolbachia* strains, as well as other intracellular bacteria such as the pathogenic *Rickettsiella* (Cordaux et al., 2007) in single isopod individuals, although most likely transient events, may have important evolutionary consequences to reshuffle bacterial genetic diversity through recombination.

Crustacean *Wolbachia* genomics

The genomes of several *Wolbachia* strains are currently being sequenced by the European consortium EuWol coordinated by Kostas Bourtzis (University of Ioannina, Greece), including the *wVulC* *Wolbachia* strain from *A. vulgare* (originating from Celles sur Belle, western France). This will represent the first genome sequence of a *Wolbachia* strain inducing feminization. We have estimated the *wVulC* genome size to ~1.7–1.8 Mb, based on pulse-field gel electrophoresis (Table 12.2). As expected for an endosymbiont, the *wVulC* genome is reduced in size compared to that of free-living bacteria. Yet, *wVulC* falls in the upper range of genome sizes estimated for various *Wolbachia* strains (~1.0 to ~1.7 Mb) (Sun et al. 2001). It is conceivable that the large size of *wVulC* for a *Wolbachia* genome is attributable, at least partly, to a high density of repetitive and mobile DNA, as previously found in the *wMel* *Wolbachia* genome from the fruit fly *Drosophila melanogaster* (Cordaux, 2008; Wu et al., 2004). In agreement with this hypothesis, it has been shown that bacteriophage WO is present in *Wolbachia* strains from several terrestrial isopods (Braquart-Varnier et al., 2005). Analysis of the *wVulC* genome sequence is expected to offer new insight into the mechanisms that allow *Wolbachia* to feminize their hosts. In this respect, the recent discovery that *wVulC* possesses genes encoding a type IV secretion system (Félix et al., 2008), which might be

Table 12.2 Sizes of DNA Fragments Produced by Digestions of the *wVulC Wolbachia* Genome with Selected Restriction Enzymes (after Félix 2004)

Enzyme	Fragment Sizes (kb)	Deduced Genome Size (kb)
Apal	550, 380, 320, 230, 180, 100	1760
AscI	910, 670, 180	1760
I-CeuI	1700 and various fragments <50 kb	~1800
FseI	No digestion	~1700–1800

involved in secretion of bacterial factors linked to *Wolbachia*-induced phenotypes (Fenn and Blaxter, 2006), represents one step closer to reaching this goal.

Feminization induction

Several studies have shown that sex determination and differentiation are very labile in isopods. Males can easily be reversed into females, and females into males, by simple experimental manipulations (Legrand et al., 1987; Legrand and Juchault, 1999). This indicates that opposite sex differentiation can be equally realized by both chromosomal determinants. Moreover, crossovers between sex chromosomes are apparently frequent and related with paucity of sex-linked genes, and unusual genetic combinations such as viable and fertile WW females or YY males. Altogether, these observations suggest that sex chromosomes must share large homologous fragments that could be considered at a primordial stage of sexual differentiation, allowing high plasticity (Juchault, 1999).

Sex differentiation in crustaceans

In *A. vulgare*, genetically male embryos (with ZZ sexual chromosomes) that harbor maternally inherited *Wolbachia* bacteria develop into functional females, which are morphologically and anatomically indistinguishable from genetic females (with ZW sexual chromosomes). This is achieved by preventing androgenic gland differentiation. In crustaceans, the androgenic gland synthesizes the androgenic hormone (AH) that is responsible for differentiation of male gonads and secondary characters. For example, androgenic gland transplantation into young *A. vulgare* females results in complete development of male gonads instead of ovaries, leading to complete sex reversal (Katakura, 1960; Juchault and Legrand, 1972). Similar results are obtained by injections of AH extracts, confirming that male differentiation is controlled by this hormone (Martin et al., 1999). AH has been purified and characterized: it is constituted of two chains linked by disulfide bridges (Martin et al., 1990; 1999; Okuno et al., 1997). AH gene expression is highly tissue-specific restricted to androgenic glands (Okuno et al., 1999; Ohira et al., 2003; Grève et al., 2004). In genetic *A. vulgare* males, AH mRNA can be detected by PCR as early as at the beginning of male gonad differentiation (unpublished results). AH thus would have an early and local action by inducing male differentiation of embryonic gonads. Therefore, feminization of genetic males that have inherited *Wolbachia* could result from inhibition of androgenic gland differentiation by targeting either the AH gene promoter or AH receptor (Juchault and Legrand, 1985).

This labile system of sex determination and differentiation suggests that genes necessary for male and female differentiation are carried by chromosomes of both sexes, in

which the W and Z chromosomes, poorly morphologically differentiated, only differ by a W-linked factor that inhibits the master gene responsible for male differentiation (Legrand et al., 1987; Juchault and Mocquard, 1993; Rigaud et al., 1997). This factor, as well as *Wolbachia*, may target the AH gene or its receptor, or another gene of the sex determination cascade, ultimately leading to inhibition of androgenic gland differentiation. This hypothesis suggests a late action of *Wolbachia* on host target during development, as opposed to very early action of other *Wolbachia* strains that induce parthenogenesis, cytoplasmic incompatibility or male killing in insects (Bourtzis and Miller, 2003).

In some cases, *Wolbachia* action may be incomplete or inefficient. For example, intersex phenotypes, ranging from fertile intersex females (iF) to sterile intersex males (iM), occur in naturally infected populations (Rigaud and Juchault, 1998). This has been interpreted as insufficient bacterial density to inhibit androgenic gland differentiation, but sufficient to target AH receptor in adults, leading to expression of partial feminization. Similar intersexes have been reported in two butterflies hosting *Wolbachia* (Kageyama and Traut, 2004; Sakamoto et al., 2007). In these species, *Wolbachia* induce early male killing, but partial feminization can be obtained with lowered bacterial burden after antibiotic treatments. Another illustration of incomplete or inefficient feminization in isopods is that naturally symbiotic males are observed in several species, such as *P. pruinosus* and *Oniscus asellus*, in which *Wolbachia* typically induce a feminizing phenotype (Marcadé et al., 1999; Rigaud et al., 1999b). Hence, low *Wolbachia* density during embryo development results in imperfect feminization or the persistence of bacteria in males, depending on the feminizing power of the strain (Bandi et al., 2001).

Wolbachia-induced feminization has also been demonstrated in two insect species, (Hiroki et al., 2002; Narita et al., 2007; Negri et al., 2006). However, host genes targeted by feminizing *Wolbachia* are different in crustaceans and insects. In insects, an interaction between bacteria and the master regulator genes that control somatic sex determination in *D. melanogaster* (*Sex-lethal* or *Doublesex* genes) has been hypothesized (Negri et al., 2006; Narita et al., 2007). Because circulating sexual hormones are lacking in insects, *Wolbachia* have to infect all host cells and interact with the genetic control of sex determination in each somatic cell. Interestingly, such local action has been reported in woodlice infected by a masculinizing virus involved in gynandromorphous mosaics (Juchault et al., 1991).

Feminization should not be considered unique to *Wolbachia*. Other symbionts, such as *Cardinium* in the mite *Brevipalpus phoenicis* (Weeks et al., 2001) and microsporidia and paramixydia in amphipods (Weedall et al., 2006; Haine et al., 2007), are able to induce genetic male individuals to develop as females. The mechanism of feminization in the mite (in which haploid symbiotic males reproduce as parthenogenetic females) is not yet known. Conversely, microsporidia-induced feminization in amphipods such as *Gammarus duebeni* or *Orchestia* spp. has been studied more extensively (Terry et al., 1999, 2004). For example, *Nosema granulosis* induces feminization in *G. duebeni* by preventing differentiation of the androgenic gland and the production of AH, which also controls male sexual differentiation in this species (Rodgers-Gray et al., 2004; Haine et al., 2007). Hence, similar pathways of sex determination may be targeted by both microsporidia and *Wolbachia*, in both amphipods and isopods.

Species-specificity of androgenic hormone and Wolbachia strains

Because feminization in isopods is likely due to bacterial factor interaction with androgenic hormone (AH) gene or its receptor, efficiency of a *Wolbachia* strain to induce feminization can be evaluated by its efficiency to inhibit AH from different host species (Juchault

and Legrand, 1985). Heterospecific implantations of androgenic glands (Table 12.3) indicated that AH from any given species exhibits high species specificity, being fully effective mainly between species of the same genus (e.g., *Oniscus*, *Porcellio*, and *Armadillidium*) (Martin and Juchault, 1999). On the other hand, Hasegawa et al. (2002) showed that a polyclonal antibody raised against recombinant precursor of *A. vulgare* AH is able to immunostain androgenic glands of six species, including *Armadillidium* and *Porcellio* species. This contrasts with inefficient graftings of *A. vulgare* androgenic glands in *P. scaber* and *P. dilatatus*, even if the reciprocal graftings induced partial masculinization (Martin and Juchault, 1999). These puzzling observations led to the investigation of AH molecular diversity in *P. scaber* and *P. dilatatus* (Ohira et al., 2003; Grève et al., 2004). Both AH shared the same overall structure with that of *A. vulgare*, but AH amino acid sequences of *Porcellio* species are only ~82% identical with that of *A. vulgare*. This high divergence may explain the failure of heterospecific androgenic gland implantations (Martin and Juchault, 1999) while remaining consistent with immunological analyses (Hasegawa et al., 2002).

Experimental horizontal transfers of *Wolbachia* between different isopod host species have been realized to test the extent of host specialization of the bacteria (Table 12.3) (review in Rigaud, 1997; Bouchon et al., 1998; Rigaud et al., 2001; Moret et al., 2001; unpublished results). Transinfection experiments showed that the effect of *Wolbachia* on isopod phenotype differed more according to host species than to bacterial phylogeny (Bouchon et al., 1998). Indeed, feminization is induced by distantly related *Wolbachia* strains (Table 12.3). By contrast, the cytoplasmic incompatibility (CI)-inducing *Wolbachia* strain from *Cylisticus convexus* also induces CI in *A. vulgare* and *P. dilatatus petiti* (Moret et al., 2001; unpublished results) even though it is closely related to the feminizing *wVulC* strain from *A. vulgare* (Bouchon et al., 1998; Cordaux et al., 2004). Finally, interspecific transfers of feminizing *Wolbachia* strains to various isopods resulted in four different outcomes in the recipient species: bacterial elimination, absence of effect, alteration of reproduction, and death of recipients (Table 12.3). Therefore, if a *Wolbachia* strain is capable of infecting different hosts, its ability to manipulate host reproduction may be limited, depending not only on the bacterial strain but also on host genetic background.

Of note is the pathogenic effect of *Armadillidium* symbionts recorded when experimentally transinfected in *Porcellio dilatatus*. In this case, massive symbiont proliferation (Juchault et al., 1974) and paralysis of the recipient (Bouchon et al., 1998) have been observed, probably involving interaction between *Wolbachia* and the host nervous system. The delay before death (53 to 150 days after *Wolbachia* transinfection) is not long enough to allow reproduction (woodlice have an annual reproductive cycle), making these hosts evolutionary dead ends for the bacteria. Interestingly, a similar phenomenon has been described in *D. melanogaster* naturally infected by the “popcorn” *Wolbachia* strain. In this case, *Wolbachia* infection succeeded because females can reproduce before death (Min and Benzer, 1997).

Although there are numerous barriers to be successfully crossed for a *Wolbachia* strain to be stably maintained in a host, evolutionary studies do demonstrate that these barriers have been crossed many times during *Wolbachia* evolution. Notably, it is of prime importance for *Wolbachia* to evade the host immune system. In *A. vulgare*, an antimicrobial peptide has been isolated (Herbinière et al., 2005) and major proteins involved in the immune system and the cytoskeleton have been recently characterized (Herbinière et al., 2008). We are currently extending this work to identify host proteins involved in response to bacterial invasions and to monitor their expression or activity with respect to *Wolbachia* infection. Such analyses are expected to provide important new insight into *Wolbachia*–host interactions.

Table 12.3 Data on Interspecific Androgenic Gland Graftings

Donors	Recipients	AG		Wolbachia Inoculation			
		Grafting	Ref.	Establishment	Transmission	Phenotype	Ref.
<i>Armadillidium nasatum</i>	<i>Armadillidium nasatum</i>	+ [+]	(3)	Yes	Yes	Fem	(5)
	<i>Armadillidium vulgare</i>	+ [+]	(5)	Yes	Yes	Fem	(4)(5)
	<i>Armadillo officinalis</i>			No	No		(4)
	<i>Helleria brevicornis</i>			Yes		No Fem	(4)
	<i>Oniscus asellus</i>			Yes	very weak	Fem/No Fem	(4)(5)
	<i>Porcellio dilatatus petiti</i>			Yes	No	Death	(4)
<i>Armadillidium vulgare</i>	<i>Porcellio scaber</i>			Yes	very weak	Fem/No Fem	(4)(5)
	<i>Armadillidium granulatum</i>			Yes		weak Fem	(1)
	<i>Armadillidium maculatum</i>	+ [+]	(5)	Yes		Fem	(1)
	<i>Armadillidium nasatum</i>	+ [+]	(5)	Yes	Yes	Fem	(1)(3)(5)
	<i>Armadillidium vulgare</i>	+ [+]	(1)(2)	Yes	Yes	Fem	(1)(3)(5)
	<i>Armadillo officinalis</i>	- [-]	(5)	No	No		(1)
<i>Armadillidium vulgare</i>	<i>Cylisticus convexus</i>	+ [-]	(5)	Yes		Fem	(4)
	<i>Eluma purpurascens</i>	- [+/-]	(5)	Yes		weak Fem	(1)
	<i>Helleria brevicornis</i>	- [-]	(5)	Yes		No Fem	(1)
	<i>Ligia oceanica</i>			Yes		No Fem	(1)
	<i>Oniscus asellus</i>	- [-]	(5)	Yes	very weak	No Fem	(1)(5)
	<i>Porcellio dilatatus dilatatus</i>	- [+/-]	(4)(5)	Yes	No	Death	(1)
	<i>Porcellio dilatatus petiti</i>			?	No	Death	(4)
	<i>Porcellio gallicus</i>	- [+]	(5)	Yes		Fem	(4)
	<i>Porcellio laevis</i>	+ [+]	(5)	Yes		weak Fem	(2)
	<i>Porcellio scaber</i>	- [+/-]	(5)	Yes	very weak	Fem/No Fem	(1)(4)(5)

<i>Chaetophiloscia elongata</i>	<i>Porcellionides pruinosus</i>	+/- [-]	(5)	?	No Fem	(4)
	<i>Sphaeroma serratum</i>			No	No Fem	(1)
	<i>Armadillidium vulgare</i>			Yes	No Fem	(2)(3)(4)
<i>Cylisticus convexus</i>	<i>Oniscus asellus</i>			Yes	Fem	(2)(4)
	<i>Porcellio scaber</i>			Yes	Fem	(2)(4)
	<i>Armadillidium vulgare</i>	- [+]	(5)	Yes	No Fem (CI)	(4)(6)
<i>Porcellionides pruinosus</i>	<i>Cylisticus convexus</i>			Yes	No Fem	(4)
	<i>Oniscus asellus</i>	- [-]	(5)	Yes	No Fem	(4)
	<i>Porcellio dilatatus dilatatus</i>	+ [+]	(5)	Yes	No Fem	(4)
	<i>Porcellio dilatatus petiti</i>			Yes	No Fem (CI)	(7)
<i>Porcellionides pruinosus</i>	<i>Porcellio scaber</i>	+ [+]	(5)	Yes	No Fem	(4)
	<i>Armadillidium vulgare</i>	- [+/-]	(5)	Yes	Fem	(2)(4)
	<i>Armadillo officinalis</i>			Yes	Fem	(4)
<i>Chaetophiloscia elongata</i>	<i>Cubaris murina</i>			Yes	Fem	(4)
	<i>Cylisticus convexus</i>	- [+]	(5)	?	Fem	(4)
	<i>Helleria brevicornis</i>			Yes	No Fem	(4)
<i>Chaetophiloscia elongata</i>	<i>Oniscus asellus</i>	- [-]	(5)	Yes	Fem	(2)(4)
	<i>Porcellio dilatatus dilatatus</i>	- [+]	(5)	Yes	Fem	(4)
	<i>Porcellio dilatatus petiti</i>			?	Fem	(4)
<i>Chaetophiloscia elongata</i>	<i>Porcellio gallicus</i>			Yes	Fem	(4)
	<i>Porcellio scaber</i>	- [+]	(5)	Yes	Fem	(2)(4)

Note: Data are from (1) Katakura, 1960; (2) Juchault and Legrand, 1972; (3) Juchault and Legrand, 1979; (4) Juchault and Legrand, 1978; (5) Martin and Juchault, 1999. (+) indicates complete masculinization; (+/-) indicates partial masculinization; (-) indicates no masculinization. [] indicates reciprocal graftings. Data on interspecific transfers of *Wolbachia* are from (1) Juchault et al., 1974; (2) Juchault et al., 1994; (3) Rigaud and Juchault, 1995; (4) Bouchon et al., 1998; (5) Rigaud et al., 2001; (6) Moret et al., 2001; (7) Grève and Johnson, unpublished. Fem, feminization; CI, cytoplasmic incompatibility; ?, individuals originally infected.

Evolutionary consequences of *Wolbachia*–isopod interactions

*Diverse sex-ratio distorters in *Armadillidium vulgare**

In *A. vulgare*, chromosomal sex determination follows a female heterogamety (i.e., ZW females and ZZ males; Juchault and Legrand, 1972). Two parasitic feminizing factors are known in this species. The first one is the *Wolbachia* endosymbiont, which is located in all tissues of females and especially concentrated in oocytes (Martin et al., 1973; Rigaud et al., 1991). Whatever their sexual genotype, all zygotes inheriting *Wolbachia* will develop a female phenotype. In this respect, ZZ males are changed into functional females which, in turn, produce female-biased broods. It has been shown that, in natural populations, all *Wolbachia*-infected females actually are ZZ individuals sexually reversed by the symbionts (Juchault et al., 1993).

By contrast, many female-biased lineages of *A. vulgare* lack *Wolbachia*. Many of the traits exhibited by these lineages are similar to those recorded in *Wolbachia*-infected lineages: females are genotypic males (i.e., ZZ) reversed by a feminizing factor termed *f* element (Legrand and Juchault, 1984). If inheritance of the feminizing effect is mainly maternal in these host lineages, sex-ratios of the progenies are very unstable, varying from all male to all female broods. Conversely, *Wolbachia*-infected host lineages produce stable female-biased broods over generations. But the main difference from lineages harboring *Wolbachia* is that the *f* element is occasionally transmitted by males with a non-Mendelian pattern. Finally, females of these lineages can be experimentally reversed into males by the implantation of an androgenic gland: resulting neo-males are able to transmit the feminizing phenotype to their offspring (Legrand and Juchault, 1984). Although the nature of the *f* element is unknown, it has been suggested that its transmission and expression are analogous to those of transposable elements or viruses. Therefore, the *f* element might be a nuclear mobile element carrying feminization capability. Following progenies of a ZW female inoculated with *Wolbachia* during five generations, Legrand and Juchault (1984) observed the spontaneous appearance of *f* occurring after *Wolbachia* had failed to be transmitted. Because lines were maintained in inbreeding, the *f* factor could not be imported through paternal inheritance. Based on the direct link between the prior infection by *Wolbachia* and the appearance of *f*, Legrand and Juchault (1984) proposed a bacterial origin for the *f* element. This hypothesis is strengthened by recent reports suggesting widespread and recurrent lateral gene transfers in *Wolbachia*–host interactions (Hotopp et al., 2007).

Theoretical predictions and intragenomic conflicts

Due to vertical transmission, survival and reproduction of intracytoplasmic symbionts are dependent on survival and reproduction of their hosts. In the context of anisogamy, endosymbionts such as *Wolbachia* are only maternally transmitted via egg cytoplasm. By inducing excess of females in populations, feminizing *Wolbachia* will consequently increase their transmission, even in the absence of any costs or advantages of the symbiosis (i.e., without selection; Werren and O'Neill, 1997). Basic population dynamics models assuming equal fecundity of both infected and uninfected females predict that such feminizers should increase in frequency in previously uninfected host populations and that asymbiotic genetic females should be replaced by symbiotic neo-females within a few generations (Caubet et al., 2000) (Figure 12.1). At equilibrium, *Wolbachia* can be considered as a cytoplasmic sex factor (CSF), as the models predict that the sex determination is only driven by presence or absence of the symbiont. *Wolbachia* transmission rate is then the only cue

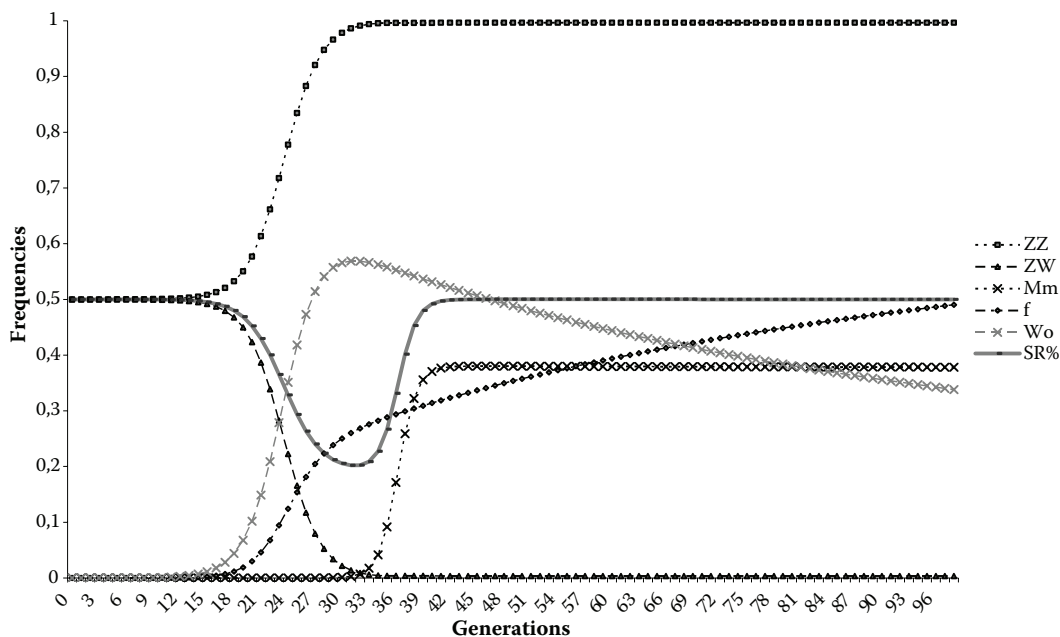


Figure 12.1 Deterministic model of evolution of sexual determinism in an isolated panmictic population infected by a feminizer. Infected ZZ females are introduced in the uninfected population at the rate of 0.001. The transmission rate of the feminizing *Wolbachia* was 0.8. The integration rate of *f* element was 0.02. ZZ = genetic males; ZW = genetic females; Mm = masculinizing gene; Wo = *Wolbachia*; *f* = *f* element; SR% = male sex-ratio (after Caubet et al., 2000; unpublished).

that drives the point where the equilibrium is reached (i.e., number of generations and frequency of feminized males).

As nuclear genes are biparentally inherited while cytoplasmic genes are only maternally inherited, CSF genes are in conflict with host autosomal genes (Werren, 1987; Caubet et al., 2000; Randerson et al., 2001). Two distinct evolutionary patterns can solve this intragenomic conflict. Because of Fisherian selection, a sex-ratio shift may be selected in uninfected lineages to compensate for female-biased sex ratios in infected lineages, leading to an overall balanced sex-ratio (Werren, 1987). Alternatively, CSF may select autosomal repressors that restore unbiased sex-ratios within infected lineages. Such repressors have been found in *A. vulgare*, acting either on the transmission rate or the expression of the feminizing elements (reviewed in Rigaud, 1997). A polygenic system of autosomal genes limiting the transmission of *Wolbachia* has been evidenced by selecting a symbiotic strain of *A. vulgare* in which females produced male-biased broods (Rigaud and Juchault, 1992). As these genes do not interfere directly with sex determination, they were referred to as “resistance” genes (*R* genes). Presence of the *f* element also leads to intragenomic conflict. Consequently, an autosomal masculinizing gene (*M* gene) which restores the male phenotype in the presence of the *f* element has been described in *A. vulgare* lineages (Rigaud and Juchault, 1993). In accordance with Fisher’s predictions, this gene is not selected in populations in which sex determination is chromosomal but the frequency of *M* increases with the *f* penetrance (Juchault et al., 1992) (Figure 12.1). The *M* gene is considered as a multifactorial sex determinant because it also overrides the female sex factor of the W chromosome (Rigaud and Juchault, 1993).

Table 12.4 Frequencies of Female Types Sampled in 10 Field Populations of *A. vulgare* from Western France (Data from Grandjean et al. 1993 and Rigaud et al. 1999a)

Populations	<i>n</i>	% Symbiotic Females	% <i>f</i> Females	% Chromosomal Females
Angoulême	11	63.6	36.4	—
Ars en Ré	46	—	95.7	4.3
Celles sur Belle 1971	18	39.0	61.0	—
1991	71	35.0	65.0	—
1993	71	35.2	64.8	—
1999	47	36.2	63.8	—
Isle-Jourdain	7	—	100	—
La Grière	30	—	100	—
Luzac	50	—	96.0	4.0
Noiziel	46	—	95.7	4.3
Saint-Cyr	35	5.7	68.6	25.7
Saint Martin du Fouilloux	7	—	100	—
Viré	25	—	100	—

Distribution in field populations and consequences

Results of these intragenomic conflicts are perceptible at the population level. Extensive surveys of *A. vulgare* populations have been conducted over 15 years (Juchault and Legrand, 1981; Juchault et al., 1993) and complemented by analyses of population structure and composition (Grandjean et al., 1993; Rigaud et al., 1999a; Verne, 2007). The combined dataset (54 populations screened) indicates that the most common sex factor present in natural populations is the *f* element, which is found in 94% of the populations (Table 12.4, examples). When present in a population, the *f* element occurs in females with a frequency ranging from 3% to 100% (Table 12.4 and data from Juchault et al., 1993). Surprisingly, the least common sex ratio distorter is *Wolbachia*. Indeed, most populations are asymbiotic (62%). In infected populations, the frequency of symbiotic females ranges from 5% to 74%, with a single exception (100%), however, based on a small sample size (Verne, 2007). Finally, the proportion of males ranges from 20% to 61% among the studied populations. These observations raise a number of questions: Why are *Wolbachia* not so widespread? How can we explain the maintenance of the female sex chromosome? Why is *f* the most frequent feminizing factor?

All symbiotic females found so far in wild populations of *A. vulgare* are genetic males reversed into functional females, supporting theoretical predictions (Juchault et al., 1993; Rigaud et al., 1999a). However, their frequency is often lower than predicted (Table 12.4). Such variation in prevalence may be explained by several nonexclusive processes such as host life history traits and host population structure. For example, infected females may suffer a fitness disadvantage relative to uninfected females. Evidence of various costs to *Wolbachia* symbiosis has already been demonstrated in terrestrial isopods which, however, are generally limited, and cannot account for the low prevalence of feminizing *Wolba-*

chia in natural populations (Rigaud and Juchault, 1998; Rigaud et al., 1999a; Rigaud and Moreau, 2004).

Wolbachia consequences in host life history traits

One of the expectations of deterministic models is the spread of infected females in the population and the correlative rarefaction of the males (Figure 12.1). Feminizing *Wolbachia* have the potential to reduce male proportion to values limiting mating possibilities and therefore limiting population size (Moreau and Rigaud, 2000). Such a situation could promote the conditions of a sexual selection. It has been shown in *A. vulgare* that *Wolbachia*-infected females (feminized males) have lower mating success and receive less sperm than asymbiotic females (Moreau et al., 2001). Males exhibit an active mate choice by interacting more and making more mounting attempts with uninfected females. At the same time, feminized males exhibit a high proportion of nonsexual behavior that stop the mating sequence. Moreover, multiple male mating induces sperm depletion that affects fertility only in infected females (Rigaud and Moreau, 2004). Moreover, multiple male mating induces a competition for sperm precedence (Moreau et al., 2002). These differences in mating rate and fertility of symbiotic females may limit the spread of *Wolbachia* and maintain infection polymorphism in populations. Furthermore, male mating capacity (i.e., the number of females that a single male can inseminate) seems correlated with the symbiotic phenotype: isopod hosts harboring feminizing *Wolbachia* have higher male mating capacity than species harboring CI *Wolbachia* (Moreau and Rigaud, 2003). This pattern may be explained either by selection of high male mating capacity following male rareness or because the feminizing phenotype would lead to population extinction if male mating capacity is not sufficient.

As a continuous supply of migrants may counterbalance male rareness, the impact of host life history traits may nevertheless be more constrained by population structure. To trace the coevolution of host and their sex-ratio distorters, host genetic differentiation was investigated in *A. vulgare* populations using mitochondrial DNA, which is maternally inherited as is *Wolbachia*. *A. vulgare* has an unusual ~42-kb-long mitochondrial genome consisting of three ~14-kb-long monomeric units (Raimond et al., 1999). The nucleotide sequence of the genome was recently determined; it showed that the three units have virtually identical sequences, thereby confirming *A. vulgare* mitochondrial DNA suitability for population genetic analyses (Marcadé et al., 2007).

Grandjean et al. (1993) and Rigaud et al. (1999a) analyzed mtDNA variation and the concomitant distribution of sex ratios distorters in French populations of *A. vulgare*. Due to its mode of inheritance, *Wolbachia* is expected to be associated to a given mitotype for a single infection event. This hitchhiking process will lead to a decrease of within-population diversity. On the other hand, association between a given mitotype and the *f* element could be broken by the possibility of paternal transmission of *f*. Both studies revealed that *A. vulgare* populations are genetically structured in correlation with *Wolbachia* prevalence. Moreover, introgression of the *f* feminizing factor into *Wolbachia* lineages was suggested. Recently, population structure was investigated using both mitochondrial DNA and nuclear microsatellite markers (Verne et al., 2006; Verne, 2007). Significant host genetic structure consistent with isolation by distance was recorded on five microsatellite loci, whereas mitochondrial DNA genetic structuration is related to *Wolbachia* prevalence (Verne, 2007). Deviations from neutrality suggest that both *Wolbachia* and other feminizing and masculinizing factors may be under frequency-dependant selection, inducing hitchhiking on host cytoplasm.

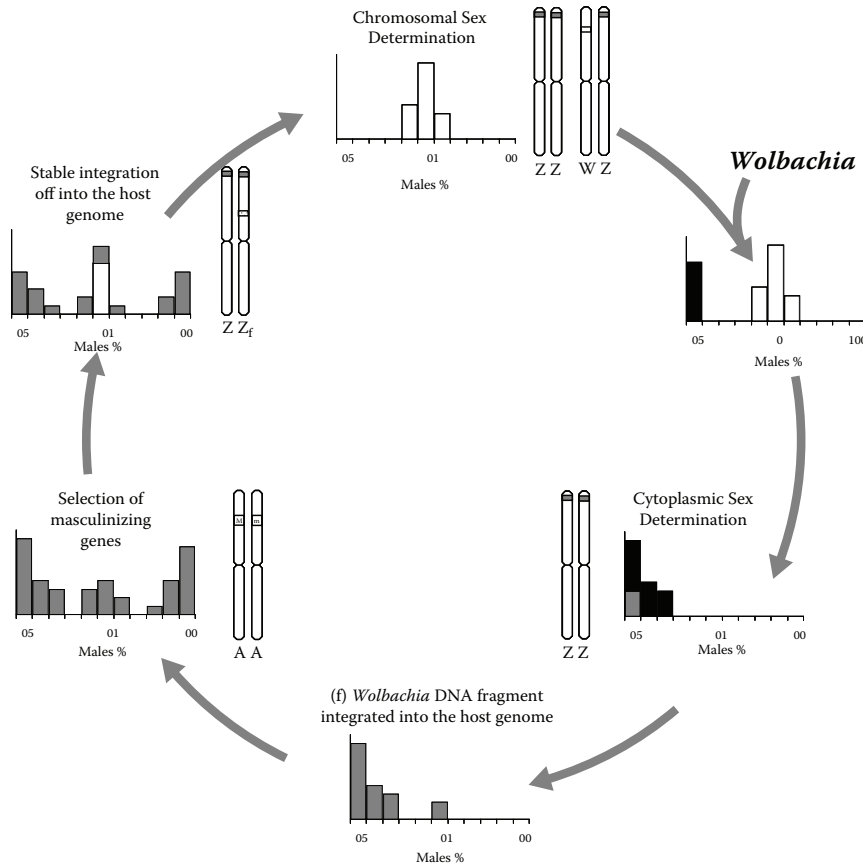


Figure 12.2 Schematic view of the evolution of sex determination and chromosomal sex determinants in *A. vulgare* (modified from Juchault and Mocquard, 1993). The symbiotic association between feminizing *Wolbachia* leads to cytoplasmic sex determination where all individuals are homogametic ZZ. Originating from *Wolbachia*, a new Mendelian sex factor (*f*) may be transferred into the host genome, leading to the selection of nuclear inhibitors (*M* genes). When stably inserted into a Z chromosome, the *f* factor generates a W-like chromosome. Distribution of progenies (male ratio) and typical chromosomal sex determinants in a given population are represented at each step of this evolutionary course. Black bars: individuals harboring *Wolbachia*. White bars: asymbiotic individuals. Grey bars: individuals harboring *f* factor.

Conclusion: dynamic evolution of sex determination in *Armadillidium vulgare*

The existence of multiple sex ratio distorters in *A. vulgare* and the occurrence of intragenomic conflicts favoring selection of host autosomal genes restoring males have led to the proposition of a dynamic view of the evolution of sex determination in *A. vulgare* (Juchault and Mocquard, 1993; Figure 12.2): this model emphasizes that the course of sex determination is governed by a subtle game between sex-ratio distorters under accelerated evolutionary circumstances and recurrent *Wolbachia* infections. The key point of such evolution is that chromosomal sex factors are not restricted to a given pair of chromosomes. There-

fore, when selected, the heterogametic masculinizing locus (*M* gene) is analogous to male heterogamety. Similarly, stable integration of a feminizing determinant (*f* element) on a Z chromosome establishes new female heterogamety. This suggests that epigenetic sex factors can repeatedly change the location of sex chromosomes along the course of this evolutionary process (Caubet et al., 1995; Caubet et al., 2000). The origin of the double heterogametic system in *Porcellio* or *Armadillidium* species and the nearly nondifferentiation of sex chromosomes in most crustaceans might be a byproduct of these recurrent intragenomic conflicts (Juchault and Rigaud, 1995).

It is often assumed that the original sex-determining system is nuclear in species that are influenced by CSF (Legrand et al., 1987; Juchault, 1999). Therefore, the evolution of sex determination in *A. vulgare* could reflect the origin of sex in crustaceans. As proposed by Juchault (1999), the appearance of separated sexes in crustaceans (and thus the appearance of sexual chromosomes) could be the evolutionary consequence of a cytoplasmic sex determination in a hermaphrodite ancestor. Such evolutionary transition from hermaphroditism to dioecy (separated sexes) has evolved more than 100 times in flowering plants (Charlesworth, 2002). Understanding sex determination systems may illuminate current theoretical ideas about the origin and evolution of sex.

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Wolbachia-induced sex reversal in *Lepidoptera*

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Introduction

It is generally assumed that insect sexes are genetically determined. In some insect species, however, sexes can be partially or completely reversed by nongenetic factors such as temperature. Notably, endosymbiotic microorganisms can affect the reproduction of their arthropod hosts in various ways, such as feminization or male killing. In two groups of lepidopteran insects (i.e., moths of the genus *Ostrinia* and the butterfly *Eurema hecabe*), it has recently been discovered that sex reversal from male to female can be caused by endosymbiotic bacteria of the genus *Wolbachia*. In this chapter, we briefly review the general mechanism of sex determination in insects and then describe the *Wolbachia*-induced sex reversal found in these two groups of lepidopteran insects. We discuss the mechanistic bases and evolutionary implications of these phenomena and attempt to integrate our knowledge of male killing and feminization, which have been recognized as distinct phenomena caused by endosymbionts.

Essence of the sex-determining mechanism in insects

Sexes are genetically determined in the majority of insects. For example, dipteran insects like the fruitfly *Drosophila melanogaster* have a male-heterogametic sex chromosome constitution, in which XX zygotes become females and XY zygotes develop into males. Lepidopteran insects like the silkworm *Bombyx mori* have a female-heterogametic chromosomal constitution, in which ZZ zygotes become males and ZW zygotes develop into females. Hymenopteran insects like the honeybee *Apis mellifera* have a haplodiploid sex-determination system, in which fertilized eggs (2n) become females and unfertilized eggs (n) develop into males (Bull, 1983; Werren and Beukeboom, 1998). The molecular mechanisms underlying sex determination and sex differentiation in the model insect *D. melanogaster* are well understood. Each cell determines its sex independently at a very early embryonic stage, and once determined, the sex of each cell is maintained during later development through a gene expression cascade consisting of *Sex lethal*, *transformer*, *doublesex* and other genes, in which sex-specific mRNA splicing plays an important role (Schütt and Nöthiger, 2000). Sex determination at a very early embryonic stage in a cell-autonomous manner is believed to be widespread among insects, on the basis that sexually mosaic individuals often occur in a wide variety of insects (Laugé, 1985). Although the molecular mechanisms of sex determination are very poorly understood in species other than *D. melanogaster*, all the sex-determining mechanisms in insects are proposed to be variations of a single model consisting of a master regulator gene (like *Sex lethal* in *D. melanogaster*) at the top of the cascade and the highly conserved *doublesex* gene at the bottom of the cascade (Figure 13.1) (Nöthiger and Steinmann-Zwicky, 1985; Bownes, 1992; Hoy, 2003). However, evidence for this general model is scarce at the present time.

Nongenetic factors affecting sex determination or sex differentiation

As stated above, sexes are basically determined by genetic factors in insects. However, in some insect taxa, nongenetic factors, such as temperature, hormonal substances, and endosymbiotic microorganisms, are believed to affect sex determination or sex differentiation

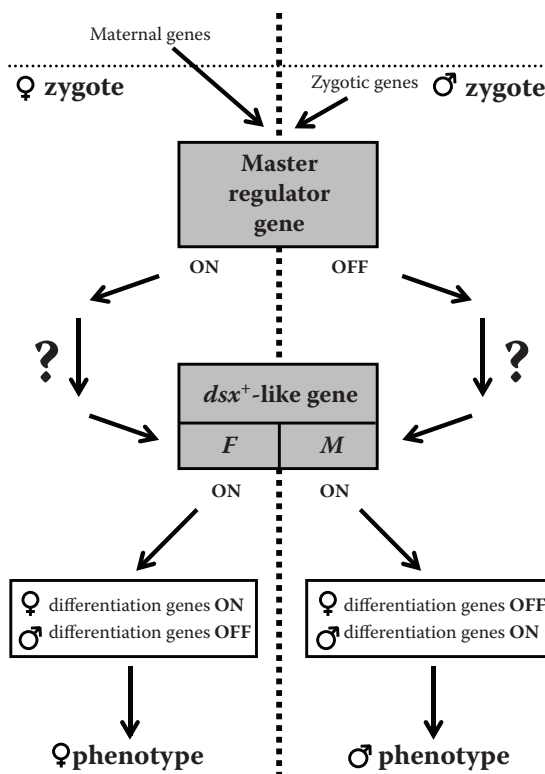


Figure 13.1 A proposed general model of sex determination in insects. This model assumes that the actions of both maternal genes and zygotic genes affect the expression of a master regulator gene, which corresponds to *Sex lethal* in *D. melanogaster*. The expression of the master regulator gene activates or suppresses the expression of subsequent genes (downstream genes). At the end of the hierarchical gene expression cascade, a highly conserved *doublesex*-like gene is subjected to alternative RNA splicing and produces the male-specific protein DSX^M or female-specific protein DSX^F. The sex-specific DSX proteins activate and suppress a series of sex-specific differentiation genes, leading to either the female phenotype or male phenotype. (Modified from Bownes, 1992).

during development or even after maturation (Bull, 1983; Werren and Beukeboom, 1998; De Loof and Huybrechts, 1998).

A switch from maleness to femaleness was reported in mosquito species of the genus *Aedes* after exposure to high temperatures (Brust, 1966, 1968; Brust and Horsfall, 1965; Horsfall et al., 1964; Horsfall and Anderson, 1961, 1965; Craig, 1965). On the other hand, a switch from femaleness to maleness after exposure to high temperatures was reported in the bagworm *Solenobia triquetrella* (Seiler, 1935) and stick insect *Carausius morosus* (Bergeard, 1958, 1961).

In the firefly *Luciferia noctiluca*, transplantation of larval male gonads into female larvae resulted in masculinization of female individuals (Naisse, 1966a, 1966b). Therefore, it has long been assumed that, unlike the majority of insects, an androgenic hormone secreted from the gonads of male larvae induces male differentiation in fireflies (De Loof and Huybrechts, 1998). Recently, the experiments carried out by Naisse in 1966 were reexamined by Maas and Dorn (2005). When larval male gonads were transplanted into female larvae,

masculinization of ovaries was never observed, and the sex of the recipient was always in accordance with the sex of its own gonads. It was therefore concluded that an androgenic hormone is not circulating in *L. noctiluca* larvae and that sex differentiation is probably regulated in the same manner as in other insect species (Maas and Dorn, 2005). At present, nothing is known about the causal agent of the masculinization of *L. noctiluca* females observed by Naisse.

De Loof and Huybrechts (1998) proposed the possible presence of a sex hormone in the tussock moth *Orgyia postica* on the basis that males exhibited higher ecdysteroid titers than females (Gu et al., 1992). At present, however, a direct causal link between ecdysteroid and sex differentiation has not been proven.

In insects, some maternally inherited microorganisms can drastically affect the sex determination (e.g., via feminization), and these effects are described in the next section.

Endosymbiotic bacteria affecting sex determination or reproduction of arthropod hosts

The reproductive systems of arthropod hosts are often manipulated by endosymbiotic bacteria such as *Spiroplasma*, *Rickettsia*, *Wolbachia*, *Arsenophonus*, and *Cardinium* (O'Neill et al., 1997; Bourtzis and Miller, 2003, 2006). Among these, *Wolbachia* are particularly focused upon due to their high prevalence (approximately 30% of insect species are infected) and the various types of reproductive manipulations they induce.

The most common type of *Wolbachia*-induced reproductive manipulation is cytoplasmic incompatibility. Cytoplasmic incompatibility results in embryonic mortality after matings between insects with differing *Wolbachia* infection statuses (Bourtzis et al., 2003), and can be either unidirectional or bidirectional. Unidirectional cytoplasmic incompatibility is typically expressed when an infected male mates with an uninfected female. The reciprocal mating is fully compatible, as are matings between infected individuals. Bidirectional cytoplasmic incompatibility usually occurs in matings between infected individuals harboring different strains of *Wolbachia* (Bourtzis and Miller, 2003). The underlying mechanism of cytoplasmic incompatibility is basically considered to be a modification-rescue system. In other words, a *Wolbachia* strain in males modifies the sperm in order to kill the offspring during embryogenesis. If the same *Wolbachia* strain is also possessed by females, the offspring will be rescued by removal of the modification (Poinsot et al., 2003; Bourtzis and Miller, 2003).

Wolbachia also induces various types of sex-ratio distortion, such as male killing, whereby male individuals (i.e., the nontransmitting sex) are selectively killed (Bourtzis and Miller, 2003), thelytokous parthenogenesis, whereby females reproduce without fertilization (O'Neill et al., 1997), and feminization, whereby genetic males are transformed into functional females (O'Neill et al., 1997; Hiroki et al., 2002; Negri et al., 2006). Feminization is likely to occur in a relatively small number of species. At present, naturally occurring feminization has only been reported in the butterfly *E. hecabe* (Hiroki et al., 2002, 2004; Narita et al., 2007a) and a leafhopper, *Zyginiidia pullula* (Negri et al., 2006). In *E. hecabe*, genetic males are completely transformed into functional females, whereas in *Z. pullula*, genetic males are incompletely feminized and exhibit deformed morphologies. Outside insects, *Wolbachia*-induced feminization is known to occur in crustacean species, such as woodlice, and has been extensively examined in *Armadillidium vulgare* (for a review, see Rigaud, 1997).

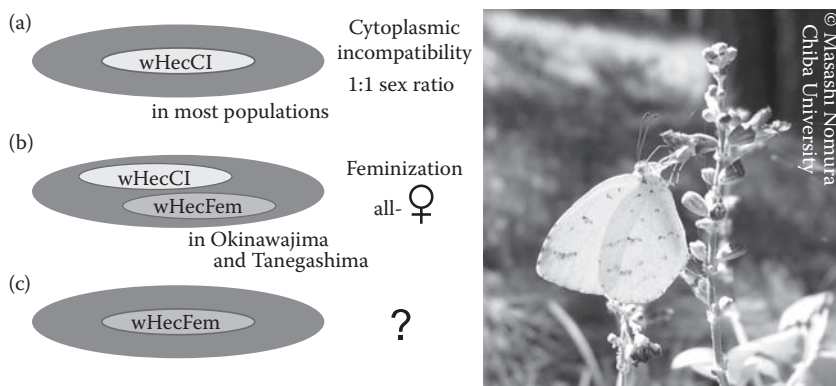


Figure 13.2 (Color figure follows p. 238.) Different *Wolbachia* infection types and their phenotypes in *E. hecabe*. (a): In most populations, butterflies singly infected with *wHecCI* exhibit cytoplasmic incompatibility. (b): In populations from Okinawajima and Tanegashima, butterflies doubly infected with *wHecCI* and *wHecFem* exhibit feminization. (c): Butterflies singly infected with *wHecFem* have never been found in natural populations or in the laboratory. Right: A female adult of *E. hecabe* in the natural condition. (Photo provided by Dr. Masashi Nomura, Chiba University.)

Wolbachia-induced feminization in the butterfly *E. hecabe*

Infection status of Wolbachia in E. hecabe

In Japanese populations of the butterfly *E. hecabe* (Lepidoptera: Pieridae), two distinct *Wolbachia* strains have been identified (Hiroki et al., 2004; Narita et al., 2007a) (Figure 13.2). One strain, designated *wHecCI* (corresponding to *wHecCI2* or *wHecFem1* in Hiroki et al., 2004), is prevalent throughout Japanese populations, except northern populations. It exhibits infection frequencies of almost 100% and causes cytoplasmic incompatibility (Narita et al., 2006; Hiroki et al., 2005). The other strain, designated *wHecFem* (corresponding to *wHecFem2* in Hiroki et al., 2004), has been detected in individuals collected in Okinawajima, one of the subtropical southwestern islands of Japan (Okinawa Prefecture), and Tanegashima, one of the temperate islands of Japan (Kagoshima Prefecture). In Okinawajima, approximately 20% of individuals are doubly infected with *wHecCI* and *wHecFem*, whereas 80% are singly infected with *wHecCI* ($n = 24$; summarized data of Hiroki et al., 2002 and 2004). In Tanegashima, approximately 90% of individuals are doubly infected with *wHecCI* and *wHecFem* ($n = 23$; Narita et al., unpublished).

Infection status of Wolbachia is associated with female-biased sex ratios

Female butterflies collected in Okinawajima and Tanegashima were individually allowed to oviposit and their offspring were reared until adult emergence. The progeny produced by females doubly infected with *wHecCI* and *wHecFem* consisted of all or nearly all females, whereas the progeny produced by females singly infected with *wHecCI* consisted of males and females at sex ratios of nearly 1:1 (Figure 13.2).

Feminization as the underlying mechanism of the female-biased sex ratios

In many lepidopteran species, including *E. hecabe*, the sex chromosome constitution is female-heterogametic (i.e., WZ females and ZZ males), and the W chromosome is cytologically observable as a condensed sex chromatin body in interphase nuclei (Traut and Marec, 1996). Cytological observations of Malpighian tubule cells and bursa copulatrix cells revealed that sex chromatin bodies were present in females of normal 1:1 sex-ratio broods, but were not observed in females of female-biased broods (Figure 13.3) (Hiroki et al., 2002; Narita et al., 2007a). These results strongly suggest that the female-biased sex ratios were caused by feminization of genetic males (ZZ). These feminized genetic males are able to copulate with normal males and produce subsequent generations that are all females.

Like many other endosymbiotic bacteria, *Wolbachia* are susceptible to tetracycline, a bacteriostatic antibiotic that inhibits bacterial growth by interfering with protein synthesis. When a tetracycline-containing honey solution was fed to adult females of female-biased broods prior to oviposition, they exclusively produced male progeny. The antibiotic treatment did not influence the 1:1 sex ratios of normal broods (Hiroki et al., 2002). Therefore, the results for antibiotic treatment (i.e., all-male production) also strongly support the notion that feminization of genetic males is the underlying mechanism of *Wolbachia*-induced female-biased sex ratios in *E. hecabe*.

Constant presence of wHecCI and frequent loss of wHecFem: implications for the population ecology of E. hecabe

The vertical transmission rates of *wHecCI* and *wHecFem* in singly infected and doubly infected matrilineages were examined. The transmission rates of *wHecCI* were nearly 100% in both singly infected and doubly infected matrilineages. The transmission rate of *wHecFem* was significantly lower than that of *wHecCI*, because approximately 20% of offspring failed to inherit *wHecFem* (Figure 13.4).

wHecCI causes cytoplasmic incompatibility with 100% intensity in *E. hecabe* (Hiroki et al. 2002, 2004). Previous studies on infection frequencies among field populations and molecular phylogeography revealed that *wHecCI* has spread rapidly from the southwest to the northeast of mainland Japan (Hiroki et al., 2005; Narita et al., 2006). The high transmission fidelity and high cytoplasmic incompatibility intensity of *wHecCI* clearly support the biogeographical data.

Although lower than *wHecCI*, *wHecFem* still has a transmission rate as high as 80%. Because nearly 100% of the offspring of doubly infected mothers are feminized, *wHecFem* has the potential to spread in host populations if at least 50% of the offspring inherit *wHecFem*. Considering the observed *wHecFem* infection frequency of 80%, it is reasonable to assume that *wHecFem* can spread and be maintained in *E. hecabe* populations. In future studies, it will be of great interest to examine the population dynamics of *E. hecabe* in populations where individuals with the two different infection types coexist.

High and stable density of wHecCI vs. low and fluctuating density of wHecFem

Although the two *Wolbachia* strains coinfect the same host insect, the cytoplasmic incompatibility-inducing strain *wHecCI* consistently exhibited 10^3 - to 10^4 -fold higher infection densities (10^{-4} – 10^{-3} copies per mitochondrial COI copy) than the feminizing strain *wHecFem* (10^{-11} – 10^{-5} copies per mitochondrial COI copy) (Figure 13.5). In a previous study, the *wHecCI* densities were consistently high and stable and the *wHecFem* densities were

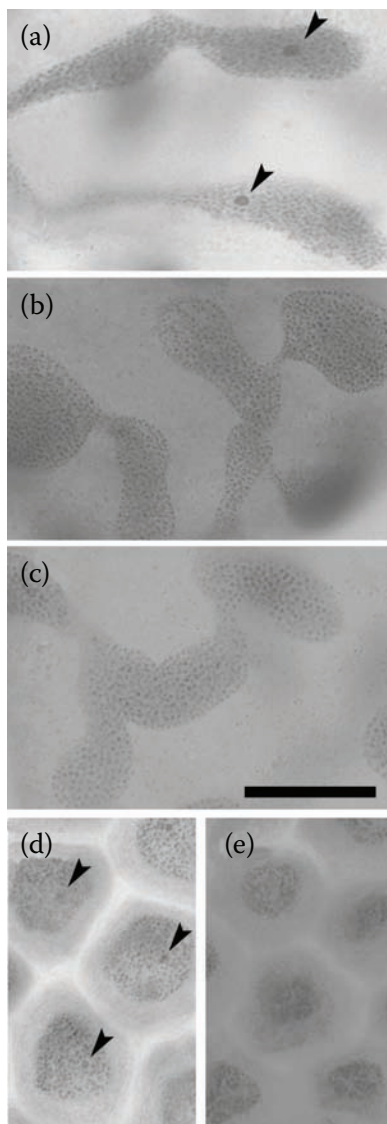


Figure 13.3 Observation of sex chromatin bodies in interphase nuclei of *E. hecabe*. (a)–(c): Nuclei of Malpighian tubule cells from a female singly infected with *wHecCI* (a), a female doubly infected with *wHecCI* and *wHecFem* (b), and a male singly infected with *wHecCI* (c). (d) and (e): Nuclei of bursa copulatrix cells from a female singly infected with *wHecCI* (d) and a female doubly infected with *wHecCI* and *wHecFem* (e). Note that the Malpighian tubule cells contain highly polyploid branched nuclei. Arrows indicate sex chromatin bodies. Bar, 20 μ m. (Adapted from Narita, S., Kageyama, D., Nomura, M., and Fukatsu, T. (2007a). *Appl. Environ. Microbiol.* **73**: 4332–4341. With permission.)

constantly low and fluctuating irrespective of the adult ages and tissues (Narita et al., 2007b). The high and stable densities of *wHecCI* may be considered as an adaptive strategy to maximize the efficiency of its vertical transmission, while the imperfect vertical transmission of *wHecFem* may be attributable to its low and fluctuating densities. The different infection densities between *wHecCI* and *wHecFem* may be relevant to their reproductive

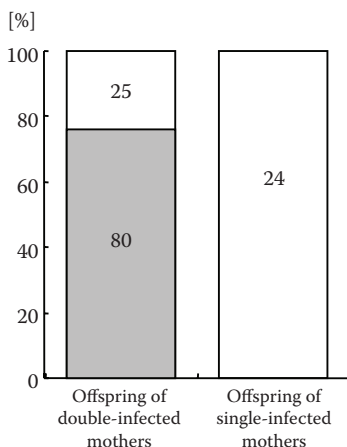


Figure 13.4 Percentages of offspring infected with each *Wolbachia* strain in the ovary. Left: Offspring of mothers doubly infected with *wHecCI* and *wHecFem*. Right: Offspring of mothers singly infected with *wHecCI*. Gray: Individuals positive for both *wHecCI* and *wHecFem*. White: Individuals positive for *wHecCI* alone. The sample size is given on each bar.

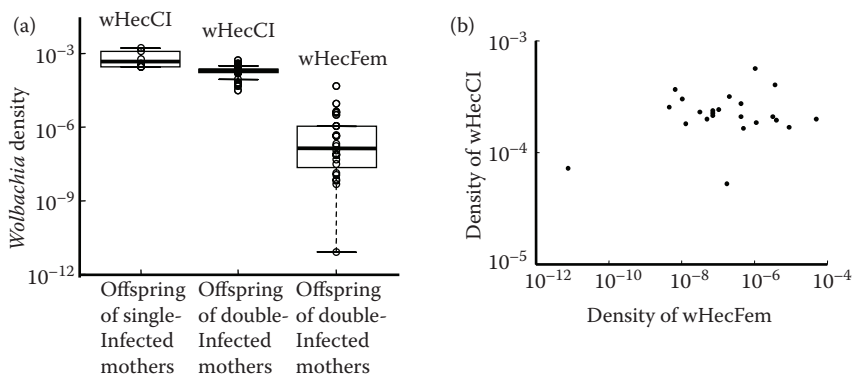


Figure 13.5 Densities of *Wolbachia* strains *wHecCI* and *wHecFem* in the ovaries of adult females examined at day 4 after adult emergence. (a): Densities of *wHecCI* in the offspring of mothers singly infected with *wHecCI* (left). Densities of *wHecCI* (middle) and *wHecFem* (right) in the offspring of mothers doubly infected with *wHecCI* and *wHecFem*. Each circle represents an individual. (b): Relationship between *wHecCI* and *wHecFem* densities within single individuals. Each dot represents an individual.

phenotypes (cytoplasmic incompatibility vs. feminization) or their different levels of adaptation to the host insect (widespread *wHecCI* vs. infrequent *wHecFem*).

Feminizing Wolbachia continuously act on E. hecabe during larval development for maintenance of female phenotypes

How and when do the *Wolbachia* endosymbionts feminize genetically male butterflies? To answer these questions, larvae were fed a tetracycline-containing diet from different

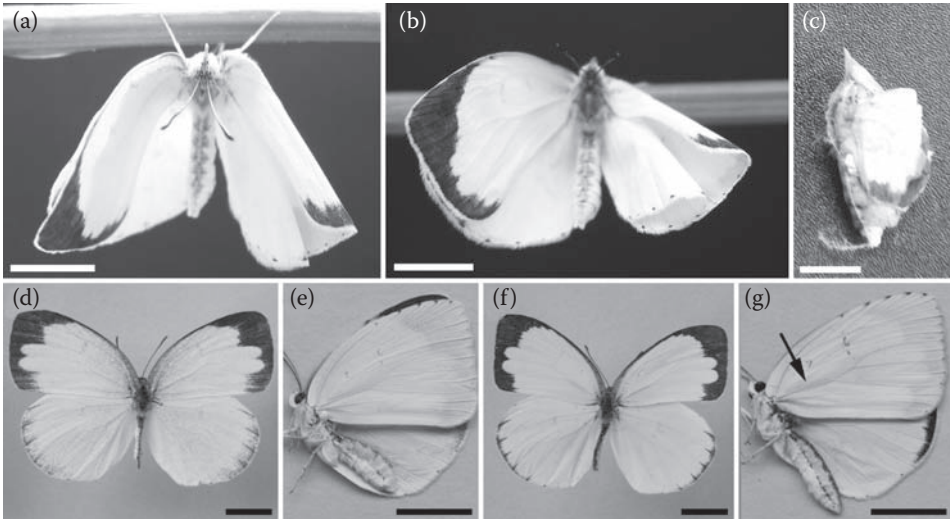


Figure 13.6 (Color figure follows p. 238.) *E. hecabe* adults that emerged after larval antibiotic treatment. (a) and (b): Emerged adult insects with deformed wings obtained after antibiotic treatment from the third to fourth instar stages of an insect line doubly infected with *wHecCI* and *wHecFem*. (c): Adult insect that failed to escape from the pupal case obtained after antibiotic treatment from the first to fourth instar stages of an insect line doubly infected with *wHecCI* and *wHecFem*. (d) and (e): Normal adult females, pale in ground color and without sex brands, representing a nontreated insect line singly infected with *wHecCI*. (f) and (g): Normal adult males, bright in ground color and with sex brands (arrows), representing a nontreated insect line singly infected with *wHecCI*. Bars, 10 mm. (Adapted from Narita, S., Kageyama, D., Nomura, M., and Fukatsu, T. (2007a). *Appl. Environ. Microbiol.* **73**: 4332–4341. With permission.)

developmental stages until pupation. When the adults emerged, most of them had wings with abnormal morphologies (e.g., curled, folded, or asymmetric) and were unable to fly. Strikingly, their wing morphologies were sexually intermediate (Figure 13.6). The expression of intersexual phenotypes in wing morphologies was strong in butterflies treated from the first instar stage, moderate in butterflies treated from third instar stage, and weak in butterflies treated from the fourth instar stage (Table 13.1). The reproductive organs and genitalia also exhibited these tendencies (Figure 13.7; Figure 13.8), because they exhibited sexually intermediate traits according to the timing and duration of the tetracycline treatment. These results strongly suggest that the sexually intermediate traits were caused by attenuated feminization due to suppression of the function of *Wolbachia* by the antibiotic treatment. Continuous infection with the feminizing *Wolbachia* during the period from the first to third instar stages appears to be required for complete expression of female phenotypes under the male genotype.

On the basis of the well-understood molecular mechanisms underlying sex determination in *D. melanogaster* (Schütt and Nöthiger, 2000) and the universal occurrence of sexual mosaicism in diverse insects (Laugé, 1985), it has been proposed that sex determination in insects generally occurs at an early embryonic stage in a cell-autonomous manner. *Wolbachia*-induced parthenogenesis makes unfertilized eggs develop into female embryos (Arakaki et al., 2001; Hagimori et al., 2006; Stouthamer, 1997), whereas *Wolbachia*-induced cytoplasmic incompatibility results in arrested embryogenesis in incompatible crosses (Bourtzis and Miller, 2003; O'Neill et al., 1997), and *Wolbachia*-induced male killing causes

Table 13.1 Sexually Intermediate Phenotypes in Wing Morphology of Antibiotic-Treated *E. hecabe*

Treated Stage	No. of Individuals with:				Total
	Feminine Color, Sex Brand 0	Masculine Color, Sex Brand 0	Masculine Color, Sex Brand ?	Masculine Color, Sex Brand 2	
From 1st instar until pupation (whole larval stage)	0	1	0	3	4
From 2nd instar until pupation	0	0	1	1	2
From 3rd instar until pupation	11	17	2	13	43
From 4th instar until pupation	3	2	1	0	6
No treatment	168	0	0	0	168

Note: Feminine color, soft yellow typical of normal females; masculine color, bright yellow typical of normal males. 0, absence of sex brand; ?, sex brand not examined or unrecognized; 2, sex brand present in both forewings. The phenotypes presented range from most feminine to most masculine (left to right).

Source: Adapted from Narita, S., Kageyama, D., Nomura, M., and Fukatsu, T. (2007a). *Appl. Environ. Microbiol.* 73: 4332–4341. With permission.

male-specific embryonic mortality (Bourtzis and Miller, 2003; Hurst and Jiggins, 2000; O'Neill et al., 1997). From these circumstantial lines of evidence, it appears natural to assume that *Wolbachia*-induced feminization should involve the transformation of genetic males into phenotypic females at an early embryonic stage. In this context, the discovery that the feminizing *Wolbachia* act continuously on the larvae of *E. hecabe* for the consummation of female phenotypes is quite unexpected and may provide some novel insights into the mechanisms underlying symbiont-induced reversal of insect sex.

Key players of feminization in E. hecabe

The strong association of *Wolbachia* infection statuses with feminization phenotypes, i.e., matrilines doubly infected with *wHecCI* and *wHecFem* exhibit feminization, whereas matrilines singly infected with *wHecCI* do not, may lead us to naïvely assume that *wHecFem* is the only causal agent of feminization. However, a more complex situation is implied by several observations as described below. *wHecCI* exhibited a high and stable density and was constantly present (transmission efficiency of 100%) irrespective of the presence or absence of *wHecFem*. In contrast, *wHecFem* exhibited an extremely low and fluctuating density and was frequently lost (transmission efficiency of 80%) (Narita et al., 2007b). Notably, offspring that spontaneously failed to inherit *wHecFem* were completely feminized (Narita et al., 2007b).

These observations may indicate that *wHecFem* does not have a feminizing effect by itself, and that factors other than *wHecFem* may act directly or cooperatively during feminization of genetically male butterflies. The host nuclear background and/or *wHecCI* can be suggested as candidates for the other factors. Because antibiotic treatment of larvae affected the sexual phenotype of feminized butterflies (Narita et al., 2007a), the *Wolbachia* strain *wHecCI* rather than the host nuclear background could play an important role in

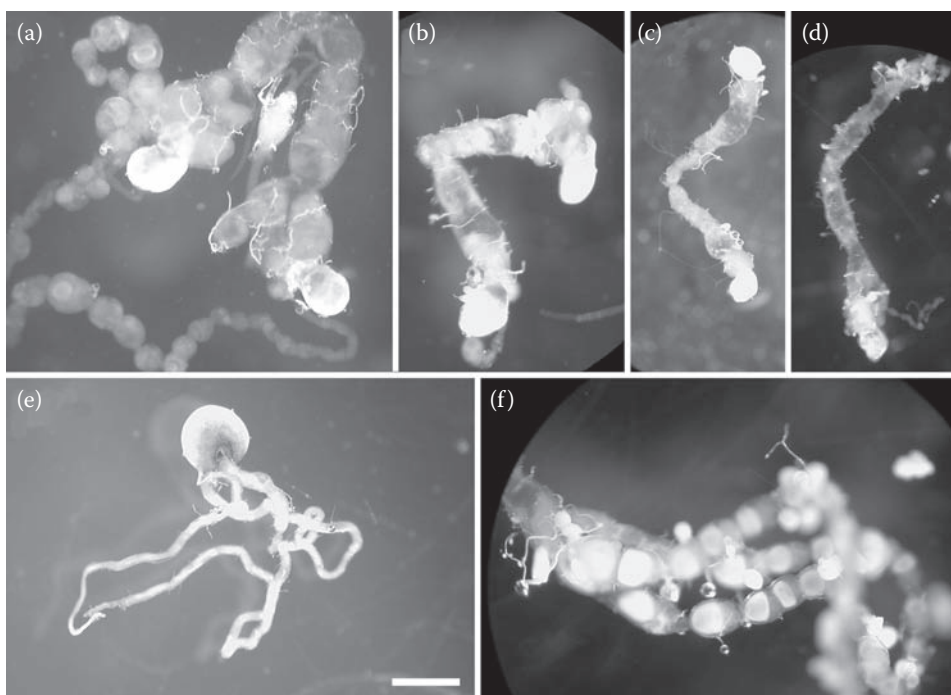


Figure 13.7 (Color figure follows p 238.) Reproductive organs of *E. hecabe* adults that emerged after larval antibiotic treatment. (a): Two deformed testes coexisting with a mature ovary obtained after antibiotic treatment from the first to fourth instar stages of an insect line doubly infected with *wHecCI* and *wHecFem*. (b) and (c): Two deformed testes obtained after antibiotic treatment from the first to fourth instar stages of an insect line doubly infected with *wHecCI* and *wHecFem*. (d): A deformed testis obtained after antibiotic treatment from the first to fourth instar stages of an insect line doubly infected with *wHecCI* and *wHecFem*. (e): A normal testis from a nontreated insect line singly infected with *wHecCI*. (f): A normal ovary from a nontreated insect line singly infected with *wHecCI*. Note that a pair of testes are often fused into one testis in lepidopteran adult insects. Arrows indicate testes. Bar, 1 mm. (Adapted from Narita, S., Kageyama, D., Nomura, M., and Fukatsu, T. (2007a). *Appl. Environ. Microbiol.* **73**: 4332–4341. With permission.)

feminization (Figure 13.9). To directly confirm this idea, the sexual phenotypes of the offspring of individuals singly infected with *wHecFem* would need to be examined. However, due to the absence of individuals singly infected with *wHecFem* in nature (Figure 13.2) and the difficulty in selectively eliminating *wHecCI* in the laboratory, this issue remains to be elucidated.

Wolbachia-induced feminizing effect and male killing in *Ostrinia* species moths

Infection status of *Wolbachia* in *Ostrinia* species

Wolbachia infection has been reported in four species in the *Ostrinia furnacalis* species complex (Lepidoptera: Crambidae), namely *O. furnacalis*, *Ostrinia scapularis*, *Ostrinia orientalis*, and *Ostrinia zaguliaevi* (Kageyama et al., 2004). Based on detailed analyses of their biological and genetic traits, it was recently proposed that *O. scapularis* and *O. orientalis* are mor-

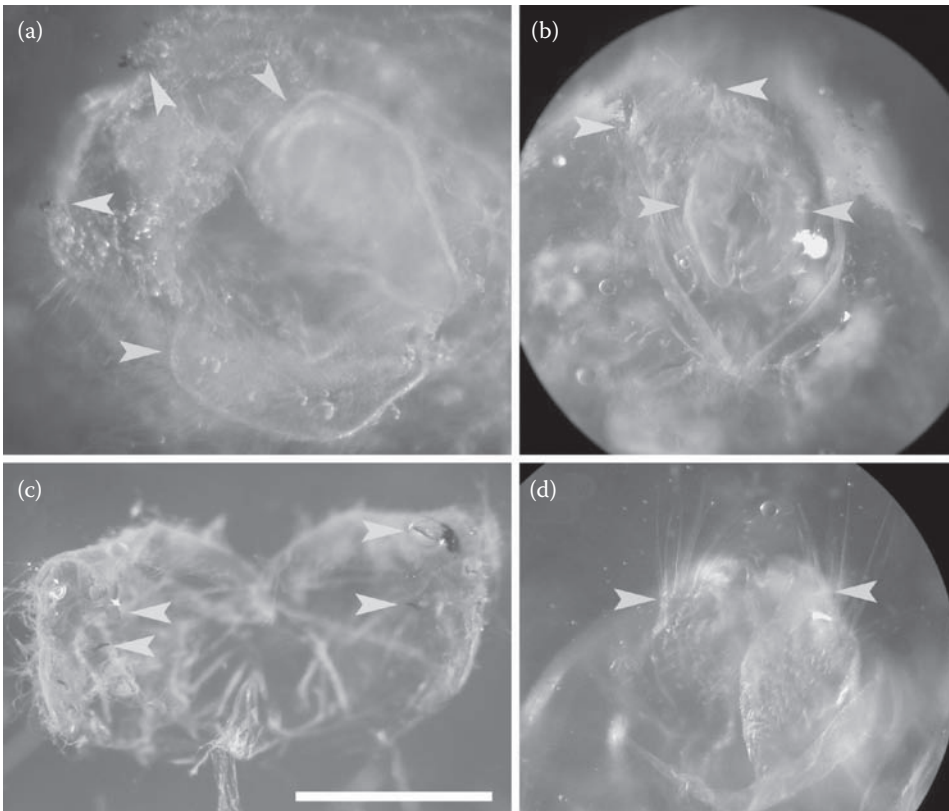


Figure 13.8 (Color figure follows p. 238.) Genitalia preparations of *E. hecabe* adults that emerged after larval antibiotic treatment. (a) and (b): Sexually intermediate genitalia obtained after antibiotic treatment from the first to fourth instar stages of an insect line doubly infected with *wHecCI* and *wHecFem*. (c): Male genitalia from a nontreated insect line singly infected with *wHecCI*. (d): Female genitalia from a nontreated insect line singly infected with *wHecCI*. Blue arrowheads indicate male traits (*bicuspid apex of valva*), and pink arrowheads indicate female traits (*papilla analis*). Bar, 1 mm. (Adapted from Narita, S., Kageyama, D., Nomura, M., and Fukatsu, T. (2007a). *Appl. Environ. Microbiol.* 73: 4332–4341. With permission.)

phological variants and form a single species designated *O. scapularis* (Frolov et al., 2007). Although *O. furnacalis* and *O. scapularis* are closely related, they are clearly distinct species that are commonly found in Japan. *O. furnacalis* mainly feeds on maize, whereas *O. scapularis* feeds on legumes and a wide range of plants. Among *O. furnacalis* and *O. scapularis*, nearly 5% of wild-caught females are infected with *Wolbachia* (Kageyama et al., 1998, 2002, 2003a) (Figure 13.10). In each of the two *Wolbachia* genes, i.e., *wsp* (555 bp) and *ftsZ* (1025 bp), DNA fragment sequences were found to be identical among different individuals within species and among different species, suggesting that they are infected with a single strain of *Wolbachia*. The *Wolbachia*-induced reproductive manipulations have been relatively well examined in *O. furnacalis* and *O. scapularis* and were found to be substantially the same. Thus, the *Wolbachia*-induced reproductive manipulations in *Ostrinia* are hereafter described for these two species.

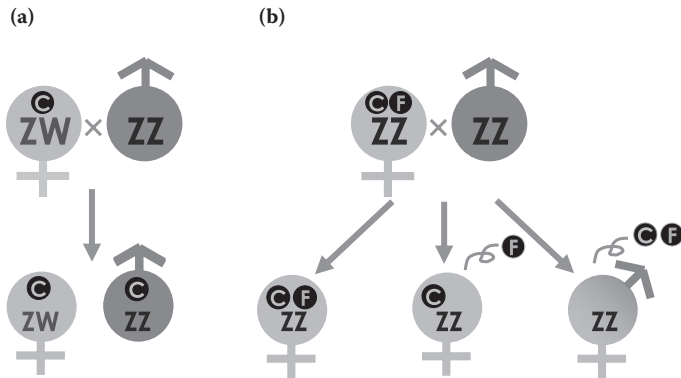


Figure 13.9 What determines the sex of *E. hecabe*? (a): Mothers singly infected with *wHecCI* produce *wHecCI*-infected offspring with 1:1 sex ratios. (b): Mothers doubly infected with *wHecCI* and *wHecFem* produce offspring consisting of all or nearly all females in the normal condition (left and middle). Approximately 80% of the offspring are doubly infected (left), while 20% of the offspring spontaneously lose *wHecFem* (middle). When offspring are treated with an antibiotic (tetracycline) during larval development, they develop as intersexes (right). These results suggest that not only *wHecFem* but also *wHecCI* may play important roles in feminizing genetic males of *E. hecabe*.

Female-biased sex ratios in Wolbachia-infected matriline

Adult females collected in six geographic locations across central and northern parts of the Honshu mainland of Japan (Figure 13.10) were individually allowed to oviposit and their offspring were reared until adult emergence. The progeny produced by *Wolbachia*-infected females consisted of all or nearly all females, whereas most of the progeny produced by uninfected females consisted of males and females with sex ratios of nearly 1:1. *Wolbachia*-infected matriline were maintained by crossing with normal males and consistently produced all or nearly all females for more than 20 generations.

Appearance of all-male progeny from mothers treated with antibiotics during larval development: possible feminization of genetic males as the underlying mechanism of the female-biased sex ratios

To investigate the effects of *Wolbachia*, *Wolbachia*-infected larvae were fed an antibiotic (tetracycline)-containing diet until pupation and found to develop into healthy female adults free from *Wolbachia* infection. Strikingly, however, the progeny produced from these cured females only consisted of males at the adult stage. These results are reminiscent of *Wolbachia*-induced feminization in *E. hecabe* (Hiroki et al., 2002; Narita et al., 2007a), and Kageyama et al. (1998 and 2002) erroneously concluded that feminization of genetic males was the underlying mechanism of the female-biased sex ratios in *Ostrinia*. However, the cytological examinations described in the next section clearly exclude the possibility of feminization as the underlying mechanism of the female-biased sex ratios.

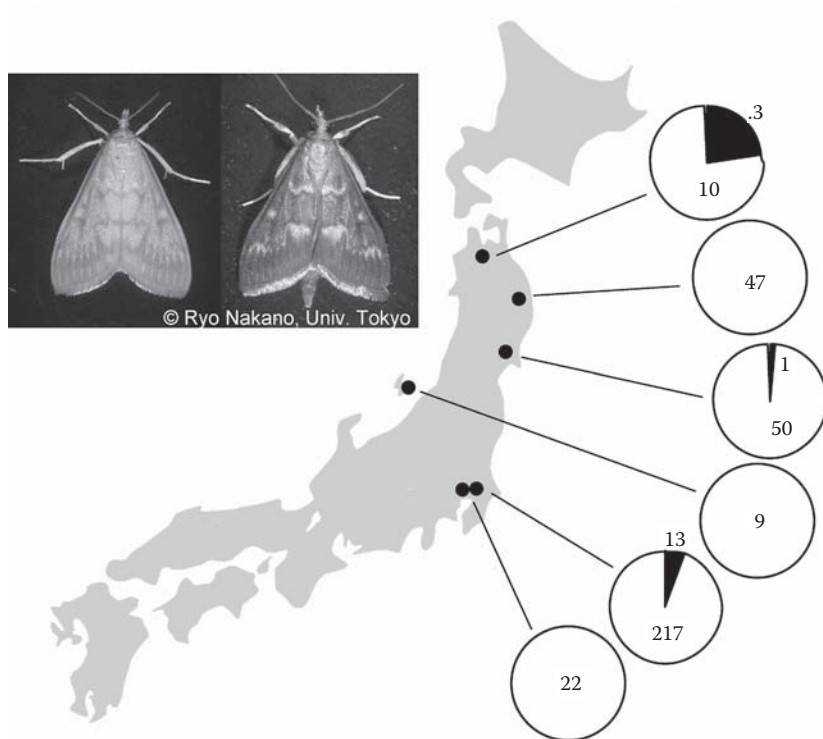


Figure 13.10 Photographs of *O. scapularis* adults (left: female; right: male), and collection sites of *O. scapularis* and *Wolbachia* infection frequencies among females in each population of *O. scapularis* (black: infected; white: uninfected; numbers indicate sample sizes). (These photographs were provided by Ryo Nakano, University of Tokyo.) (Data from Kageyama, D., Nishimura, G., Hoshizaki, S., and Ishikawa, Y. (2003a). *Genome* **46**: 974–982.)

Cytological observations reveal that feminization is not the underlying mechanism of the female-biased sex ratios

Cytological observations of sex chromatin bodies (condensed W chromosome in highly polyploid interphase nuclei) in Malpighian tubule cells and bursa copulatrix cells are often useful for clarifying the sex chromosome constitution (WZ in females; ZZ in males) (Traut and Marec, 1996). In *Ostrinia*, all the *Wolbachia*-infected mothers that produced female-biased progeny and their daughters had the WZ karyotype. All the uninfected mothers that produced progeny with 1:1 sex ratios also had the WZ karyotype (Kageyama and Traut, 2004). These results indicate that the sex ratio distortion found in this species is not due to feminization.

Male killing when Wolbachia is present

Larvae at the hatching stage were examined for the presence or absence of sex chromatin bodies to identify their genetic sexes (WZ or ZZ). Some larvae, which apparently developed well but did not leave the eggshell, were regarded as unhatched. In broods from *Wolbachia*-infected mothers, the WZ:ZZ ratios of unhatched larvae were significantly biased toward

Table 13.2 WZ:ZZ Ratios of Larvae, Inferred from Sex-Chromatin Status

Mother	Larvae at Hatching Stage			Last-Instar Larvae ^a WZ:ZZ
	Not Hatched W Z:ZZ	Hatched W Z:ZZ	Hatched plus Not Hatched WZ:ZZ	
Uninfected	14:20	31:31	45:51	35:32
Infected	12:30*	46:33	58:63	40:0**
Cured	54:3**	16:72**	70:75	0:26**

^a In the last instar, all WZ larvae were females and all ZZ larvae were males according to the gonad anlagen.

* X²-test, deviation from the 1:1 ratio significant ($P < 0.01$)

** X²-test, deviation from the 1:1 ratio significant ($P < 0.001$).

Source: Adapted from Kageyama, D. and Traut, W. (2004). *Proc. R. Soc. Lond. B.* **271**: 251–258.

ZZ (i.e., genetic males). The WZ:ZZ ratios of the survivors increased with larval development and finally reached 1:0 (i.e., genetic females only) at the last instar stage (Kageyama and Traut, 2004; Sakamoto et al., 2007) (Table 13.2). These results clearly indicate that *Wolbachia* kills genetic males (ZZ individuals) during larval development and that *Wolbachia* infection is compatible with the development of genetic females (WZ individuals). These findings indicate that *Wolbachia* in *Ostrinia* is a male killer and does not feminize ZZ individuals into functional females.

Female killing when Wolbachia has been eliminated

In contrast, the WZ:ZZ ratios of unhatched larvae in broods from cured mothers were significantly biased toward WZ (i.e., genetic females) (Kageyama and Traut, 2004; Sakamoto et al., 2007). The WZ:ZZ ratios of the survivors decreased with larval development and finally reached 0:1 (i.e., genetic males only) at the last instar stage (Kageyama and Traut, 2004; Sakamoto et al., 2007) (Table 13.2). These results clearly indicate that, when mothers are cured of *Wolbachia* infection by antibiotic treatment during larval stages, genetically female offspring (WZ individuals) die during larval development while male offspring (ZZ individuals) survive.

There are two hypotheses that can account for the female-killing mechanism, which we refer to as the compensation hypothesis and the modification-rescue hypothesis (Figure 13.11). The compensation hypothesis assumes host genetic differences in maternally inherited factors (i.e., cytoplasmic elements or W-linked genes) between infected and uninfected matriline. Due to this difference, infected matriline lack some essential genetic factors necessary for the early development of females, but *Wolbachia* does compensate for these factors. In other words, this hypothesis assumes a historical coevolution between the bacteria and the hosts. In contrast, the modification-rescue hypothesis does not assume host genetic differences between infected and uninfected matriline. *Wolbachia* are assumed to modify *Ostrinia* maternally in order to kill the daughters. Furthermore, the transmitted *Wolbachia* are assumed to rescue the modified daughters. In other words, daughters of infected mothers are rescued and can survive if *Wolbachia* have been successfully transmitted. Otherwise, they are killed by the effect of the modification.

These two hypotheses are mutually exclusive and testable by transfection of *Wolbachia*. If female-biased matriline are established by transfecting *Wolbachia* from infected matriline into uninfected matriline, all we need to do is eliminate the *Wolbachia* infection from

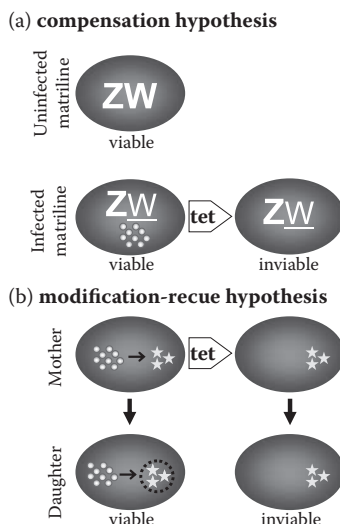


Figure 13.11 Two hypotheses can account for the female-killing mechanism when *Wolbachia* have been removed from mothers. (a): The compensation hypothesis assumes that infected matrilines lack some matrilineal genetic factors (i.e., W-linked or cytoplasmic factors) that are necessary for the survival of daughters, although the autosomal genes of infected and uninfected matrilines are homogeneous. This hypothesis also assumes that *Wolbachia* compensate for the deficiency of the matrilineal trait of infected matrilines. (b): Instead of assuming genetic differences, the modification-rescue hypothesis assumes that *Wolbachia* modify mothers in order to selectively kill the daughters (maternal imprinting). This hypothesis also assumes that inherited *Wolbachia* (i.e., those successfully transmitted to daughters) rescue the daughters from being killed.

the newly established matrilines and examine the sex ratios of their offspring. If the sex ratios are 1:1, the compensation hypothesis would be correct. In contrast, if the sex ratios are male-biased, the modification-rescue hypothesis would be correct.

Antibiotic treatment of adult females leads to the production of progeny with intersexual phenotypes

When mothers were fed an antibiotic-containing sucrose solution during the adult stage prior to oviposition, a considerable number of offspring with intersexual phenotypes appeared (Figure 13.12) (Kageyama et al., 2003b; Kageyama and Traut, 2004). Eggs laid during the first to third days after tetracycline treatment developed as females only. Eggs laid on the fourth and fifth days developed as females, intersexes, or males. Eggs laid from the sixth to ninth days developed as males only (Table 13.3). The successive appearance of females, intersexes, and males suggests that eggs laid early after treatment onset were still under the influence of the *Wolbachia* infection, whereas eggs laid 4–5 days after treatment onset were partly cured of the *Wolbachia* infection, and those laid from day 6 onwards were completely cured. Cytological observations of Malpighian tubules, testes, and bursa copulatrix cells revealed that phenotypic females were genetically female (WZ), phenotypic males were genetically male (ZZ), and all intersexual individuals were genetically male (ZZ) in all tissues. Strikingly, the bursa copulatrix, which is a female-specific organ, had the male genotype (ZZ). In other words, this organ had the female phenotype under

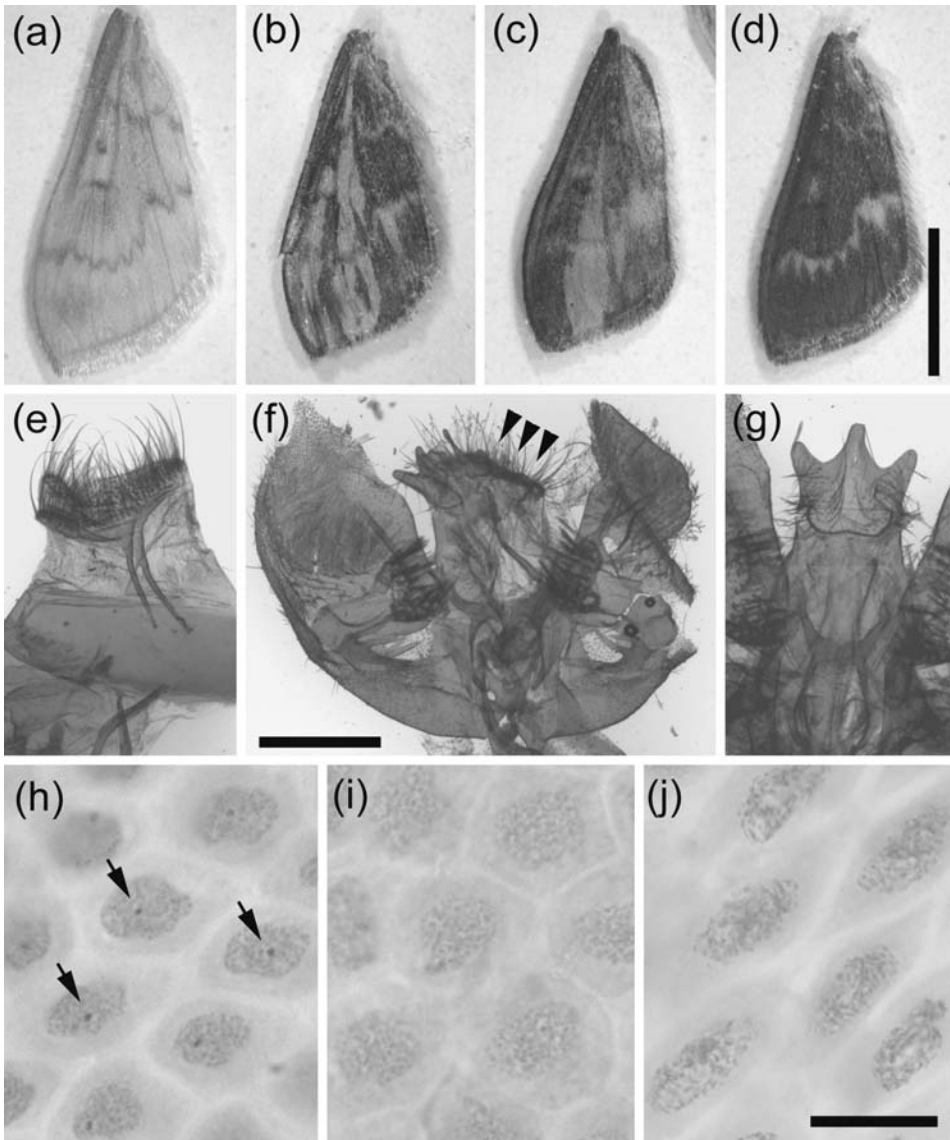


Figure 13.12 Morphological and cytological features of intersexual individuals. (a): Left forewing of an uninfected female. (b): An intersexual individual generated by transfection. (c): An intersexual individual generated by tetracycline treatment. (d): An uninfected male. (e)–(g): External genitalia of an uninfected female (e), an intersexual individual generated by transfection (f) and an uninfected male (g). (h)–(k): Interphase cells of the bursa copulatrix from an uninfected female (h), an intersexual individual generated by transfection (i), and an intersexual individual generated by tetracycline treatment (j). Ovipositor and ovipositor-like structures; uncus and uncus-like structures; sex chromatin bodies. The magnifications of (a)–(d), (e)–(g), and (h)–(j) are equal. Scale bars: 5 mm (a–d); 0.5 mm (e–g); 20 mm (h–j). (Adapted from Kageyama, D. and Traut, W. (2004). *Proc. R. Soc. Lond. B.* **271**: 251–258.)

Table 13.3 Fates of Successive Egg Batches Laid by a *Wolbachia*-Infected Female after Tetracycline Treatment

Day of Oviposition (after Treatment)	Eggs Laid	Adults Eclosed		
		Females	Intersexes	Males
1	24	8	0	0
2	30	5	0	0
3	32	6	0	0
4	44	6	9	5
5	28	2	1	5
6	46	0	0	19
7	20	0	0	8
8	9	0	0	4
9	15	0	0	5

Source: Adapted from Kageyama, D., Ohno, S., Hoshizaki, S., and Ishikawa, Y. (2003b). *Genome*. **46**: 983–989. With permission.

the male genotype (i.e., feminization). Intersexual individuals were also generated in *O. scapularis* after transfecting *Wolbachia* from an infected matriline to an uninfected matriline (Kageyama and Traut, 2004). These intersexual individuals were not genetic mosaics but genetically homogeneous male individuals. In this sense, the fundamental mechanism of intersexual development in *Ostrinia* species is likely to be the same as that in *E. hecabe*.

Integrated explanation of the mechanism underlying male killing

As shown above, *Wolbachia* cause male killing in *Ostrinia*. It is obvious from the generation of intersexual individuals with the male genotype that *Wolbachia* have a feminizing effect on genetic males. How can we reconcile these seemingly distinct phenomena of male killing and feminization? We consider that, in *Ostrinia*, the fully expressed feminizing effect of *Wolbachia* is lethal to genetic males, whereas the weakly expressed feminizing effect can be nonlethal to genetic males. We therefore propose that the intersexual individuals obtained after antibiotic treatment of mothers could have survived because they were only partially feminized (Figure 13.13).

Mechanistic bases of male killing and feminization

Does Wolbachia interfere with sex-determining genes?

Both *Wolbachia* in *Ostrinia* and *Wolbachia* in *E. hecabe* have feminizing effects on their genetic male hosts. Thus, it is natural to assume that *Wolbachia* manipulates genes among the sex-determining gene cascades of their hosts. Unlike *D. melanogaster* (Baker et al., 1987; Schütt and Nöthiger, 2000), the sex-determining mechanisms are not well understood in lepidopteran insects, except for the fact that *doublesex* (*dsx*) gene expression is sex-specifically spliced in the silkworm *B. mori* (Ohbayashi et al., 2001; Suzuki et al., 2003, 2005, 2008). By investigating the splicing patterns of the *dsx* gene expression in normal males, normal females, feminized individuals, and intersexual individuals of *E. hecabe* and

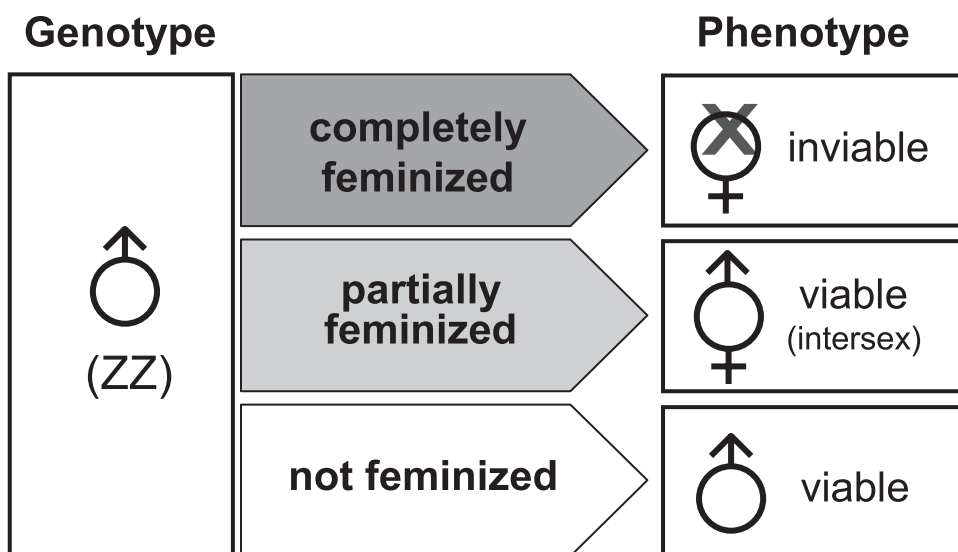


Figure 13.13 Proposed mechanism of male killing in *Ostrinia*. Upper: Under complete influence of *Wolbachia*, genetic males are completely feminized into phenotypic females but are inviable due to some forms of incompatibility between the genotypic sex and the phenotypic sex. Middle: Under incomplete influence of *Wolbachia* (i.e., by incomplete curing of *Wolbachia* infection from mothers or transfection of *Wolbachia* into uninfected eggs), genetic males are partially feminized and develop into intersexes, at least some of which are viable. Bottom: In the absence of *Wolbachia*, genetic males develop into phenotypic males.

Ostrinia, we may be able to clarify whether the target of *Wolbachia* is upstream or downstream of *dsx* within their sex-determining gene cascades (Figure 13.14). Future elucidation of the whole sex-determining mechanism in *B. mori* will provide strong reference information when investigating the effects of *Wolbachia* on the sex-determining mechanisms in *Ostrinia* and *E. hecabe*.

Does Wolbachia interfere with dosage compensation?

Organisms with male heterogametic (XX in females and XY in males) or female heterogametic (WZ in females and ZZ in males) sex-determining systems have different numbers of X chromosomes (or Z chromosomes) between males and females. To equalize the titers of X-linked (or Z-linked) gene expression between males and females, many organisms adopt two alternative processes: overexpression of X-linked genes in males or underexpression of X-linked genes in females (Marín et al., 2000; Parkhurst and Meneely, 1994; Charlesworth, 1996). These mechanisms are collectively referred to as dosage compensation. It is known that *Drosophila*, *Caenorhabditis elegans*, and mammals undertake dosage compensation. In the lepidopteran insect *B. mori*, however, dosage compensation is absent, because Z-linked gene expression is twofold higher in males than in females (Suzuki et al., 1998, 1999). Therefore, in *B. mori*, males and females function normally despite the unequal amounts of Z-linked gene products between males and females.

In *D. melanogaster*, the endosymbiotic bacteria *Spiroplasma* are known to cause male killing (Williamson and Poulson, 1979). A male-specific protein complex (dosage compensation

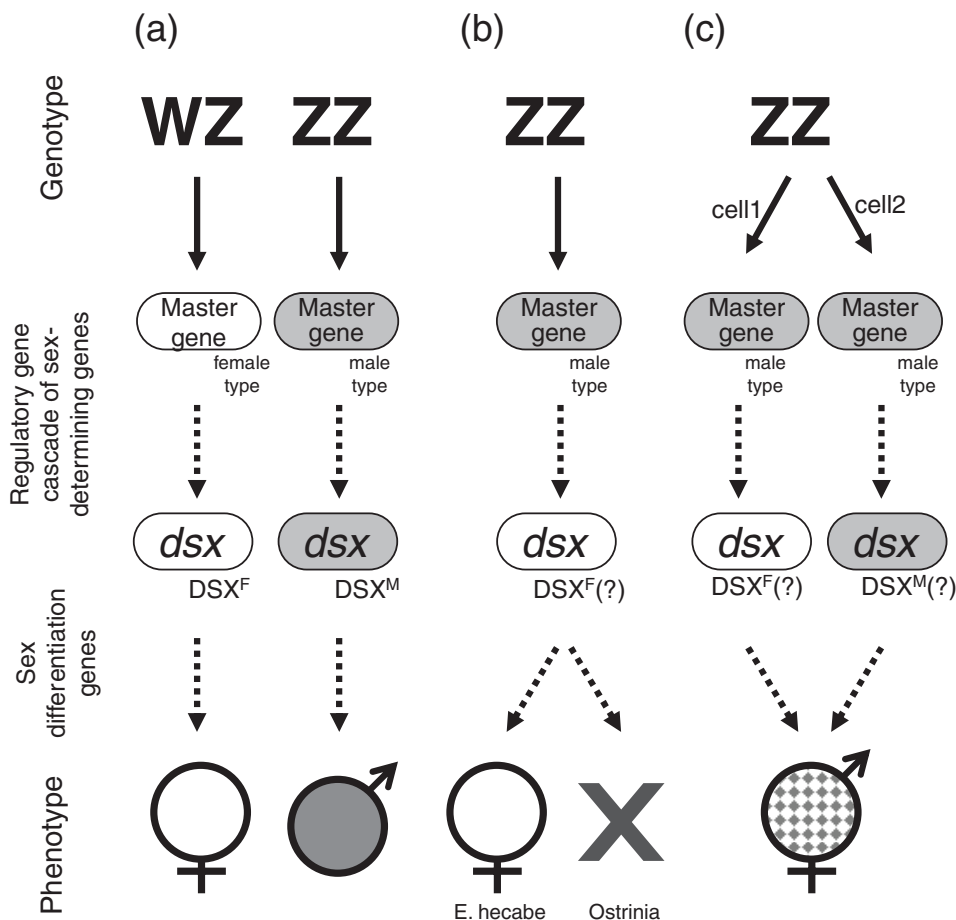


Figure 13.14 Hypothetical molecular mechanisms for sex determination in *E. hecabe* and *Ostrinia*. (a): In uninfected matriline, morphological sexual dimorphism is generated by proper expression of sex-determining and sex-differentiation genes according to their genetic sexes. (b): In feminized matriline, the expression of one of the sex-determining or sex-differentiation genes is switched from the male-type to the female-type at a particular point. In the downstream of this point, the gene expressions are consistently of the female type. Consequently, phenotypically female adults are generated under the male genotype in *E. hecabe*, whereas genetic males die during larval development in *Ostrinia*. (c): Intersexual individuals generated in *E. hecabe* and *Ostrinia* are purely genetic males, but are comprised of mosaics of phenotypically female and male tissues.

complex: DCC), which is necessary for dosage compensation, was found to be required for the expression of *Spiroplasma*-induced male killing (Veneti et al., 2005).

The death of *Ostrinia* genetic males is considered to be due to their intolerance of the feminizing effect of *Wolbachia* (Kageyama and Traut, 2004). This may imply that genetic males (ZZ individuals) cannot survive as phenotypic females due to the adverse effects of the excessive expression of Z-linked genes. Therefore, why do feminized genetic males of *E. hecabe* survive and function normally? To answer or validate this question, we need to

examine the Z-linked gene expression levels among normal females, normal males, and feminized individuals of *E. hecabe* and *Ostrinia*.

Wolbachia genotype or host genotype: which is responsible for the reproductive phenotype?

Wolbachia exhibit various types of reproductive manipulations in their hosts. Which genotype is responsible for the types of reproductive manipulations, the *Wolbachia* genotype or the host genotype? Fujii et al. (2001) transferred *Wolbachia* from *O. scapularis* into the moth *Ephesia kuehniella*, which was previously cured of a naturally occurring *Wolbachia* infection. A newly established *Wolbachia*-infected matriline of *E. kuehniella* exhibited male killing (i.e., female-biased sex ratios, egg hatch rates of nearly 50%, and 1:1 sex ratios following tetracycline treatment). Because *Wolbachia* was assumed to cause feminization in *Ostrinia* at that time, this result was considered to indicate that the types of reproductive manipulations, i.e., feminization and male killing, are attributable to host genetic differences between *Ostrinia* and *Ephesia*. Because naturally occurring *Wolbachia* in *Ostrinia* actually cause male killing instead of feminization, this result does not support the assumption that host genetic differences determine the types of reproductive manipulation.

There is a convincing case in which the host genetic background is responsible for the types of reproductive manipulations. The almond moth *Cadra cautella* is doubly infected with two *Wolbachia* strains, *wCauA* and *wCauB*, and expresses strong cytoplasmic incompatibility. Tetracycline treatment generated a *C. cautella* strain singly infected with *wCauA* and this strain was found to express strong cytoplasmic incompatibility by itself (Sasaki et al., 2005). *Wolbachia* were artificially transferred from *C. cautella* into *E. kuehniella* and an *E. kuehniella* strain singly infected with *wCauA* was generated. All-female production, egg hatch rates of 50%, and 1:1 sex ratios following tetracycline treatment showed that the *wCauA* strain expressed male killing in the *E. kuehniella* host (Sasaki et al., 2002). In other words, *wCauA* caused cytoplasmic incompatibility in *C. cautella* and male killing in *E. kuehniella*.

In the flour beetle *Tribolium confusum*, cytoplasmic incompatibility is caused by a naturally occurring *Wolbachia* strain, *wCon* (Fialho and Stevens, 1997). It is interesting that, in the closely related species *Tribolium madens*, a naturally occurring *Wolbachia* strain indistinguishable from *wCon* by DNA sequencing of the *wsp* and *ftsZ* genes causes male killing (Fialho and Stevens, 2000). Although it is unclear whether the *Wolbachia* genome or the host genome is responsible for determining the type of reproductive manipulations in *Tribolium*, these insects may represent an ideal system for investigating the mechanisms of *Wolbachia*-induced reproductive manipulations.

Although not technically easy, reciprocal transfection of *Wolbachia* strains between various insects that exhibit different reproductive manipulations, such as *Ostrinia* and *E. hecabe*, may greatly contribute to clarifying the important issue of whether the types of reproductive manipulations are determined by the *Wolbachia* genotype or the host genotype, or both genotypes. Clarification of this issue will lead to future understanding of the mechanisms of *Wolbachia*-induced reproductive manipulations.

Evolutionary implications of male killing and feminization

To date, male killing has been reported in various species of insects, including fruitflies, mosquitoes, butterflies, moths, ladybird beetles, and parasitic wasps. Furthermore, the

causal agents of the male killing belong to taxonomically diverse microorganisms, such as bacteria belonging to *Wolbachia*, *Rickettsia*, *Arsenophonus*, *Spiroplasma*, and *Flavobacterium* and unicellular prokaryotes belonging to Microsporidia (Hurst and Majerus, 1993; Hurst and Jiggins, 2000; Hurst et al., 2003). Therefore, male killing is considered to be a trait that is easy to evolve (Hurst et al., 2003). On the other hand, endosymbiont-induced complete feminization has only been reported in *E. hecabe* among insects. Even among all arthropods, microbe-induced feminization has only been found in a few species, such as woodlice and shrimps (Rigaud, 1997; Dunn et al., 1993). Despite its rare occurrence, feminization is a more advantageous strategy for maternally transmitted endosymbionts than male killing, because all the offspring of infected mothers can transmit the infection to subsequent generations in the case of feminization, whereas only half the offspring can transmit the infection to subsequent generations in the case of male killing.

The male killing observed in *Ostrinia* is considered to be death of genetic males due to the feminizing effect of *Wolbachia*. Some endosymbionts may have a feminizing effect on genetic male hosts and this effect can often be lethal. In *E. hecabe*, this feminizing effect may somehow be nonlethal, such that genetic males completely revert to functional females. Evolutionary transitions in either the host (*E. hecabe*) or the endosymbiont (*Wolbachia*) can account for the nonlethal complete feminizing effect on genetic males of *E. hecabe*, i.e., *E. hecabe* may have evolved a trait in genetic males such that they are not killed by feminization or *Wolbachia* may have evolved a trait not to kill genetic males while feminizing them.

Overall, it is undoubtedly the case that male killing and feminization are both outcomes of the close associations between endosymbionts and the sex-determining systems of their hosts. By untangling such complex interactions between endosymbionts and their hosts, we may be able to reveal unknown aspects of sex determination or sex differentiation in insects.

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Wolbachia and Anopheles mosquitoes

Jason L. Rasgon

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The problem of malaria

Human malaria, caused exclusively by *Plasmodium* parasites, infects up to 500 million people and results in almost 3 million deaths per year (Hay et al., 2004). The malaria parasites are dependent on *Anopheles* mosquitoes for transmission between human hosts (Collins and Paskewitz, 1995). Control of the disease is currently limited to antiparasitic drugs and mosquito control (Beaty, 2000) and is hampered by the evolution of drug and insecticide resistance (Talisuna et al., 2004; Hemingway and Ranson, 2000). Thus, there has been a recent concerted effort to develop genetically modified *Anopheles* mosquitoes that are unable to transmit *Plasmodium* (Ito et al., 2002; Marrelli et al., 2007).

Before deployment of genetically modified mosquitoes for malaria control can be implemented, three critical milestones must be met. These include engineering gene effectors that block pathogen transmission in the mosquito, integration and expression of effectors in the mosquito genome, and spread of the transgene into natural mosquito populations (Rasgon and Scott, 2003; Rasgon and Gould, 2005; James, 2005). Although there has been significant progress toward the first two items (Ito et al., 2002; Marrelli et al., 2007), there is no available drive mechanism to spread transgenes into natural *Anopheles* populations.

Wolbachia

One such potential transgene driver is the endosymbiont *Wolbachia* (Rasgon and Scott, 2003; James, 2005). In mosquitoes, the maternally inherited symbionts are generally associated with cytoplasmic incompatibility (CI)—reduced egg hatch in matings between uninfected females and infected males. Matings between infected females and infected

or uninfected males are fertile. Thus, infected females have a reproductive advantage, which, coupled with maternal inheritance, can cause *Wolbachia* to spread rapidly through host populations. Model predictions of *Wolbachia* dynamics have been validated in cage experiments (Curtis, 1976; Xi et al., 2005) and by observations of the dynamics of *Wolbachia* infections in natural insect populations (Turelli et al., 1992; Turelli and Hoffmann, 1995; Rasgon and Scott, 2003). If a transgene is inserted into the *Wolbachia* genome, or placed on a separate maternally inherited construct, the transgene will “hitchhike” with the symbiont into the population (Figure 14.1), replacing the natural population with one that is refractory to parasite transmission (Turelli and Hoffmann, 1999).

An alternative *Wolbachia*-based malaria control strategy is to reduce mosquito population levels by releasing *Wolbachia*-infected (i.e., incompatible) males into uninfected natural populations. In this scenario, released males are reproductively incompatible with the wild females, resulting in sterility. This strategy is functionally equivalent to the sterile insect technique (SIT), but with the advantage that males do not need to be exposed to damaging radiation or chemosterilants that might lower their mating competitiveness (Arunachalam and Curtis, 1985; Shahid and Curtis, 1987; Dobson et al., 2002a; Zabalou et al., 2004; Brelsfoard et al., 2008).

The third strategy is to release mosquitoes infected with virulent *Wolbachia* strains that shorten mosquito life span. After feeding on an infected host, a mosquito must survive for a period of up to 2 weeks before it is able to transmit the parasites. Thus, the daily probability of survival is the most sensitive component of a vector’s role in pathogen transmission (Garrett-Jones, 1964; Rasgon et al., 2003). Control strategies that reduce mosquito

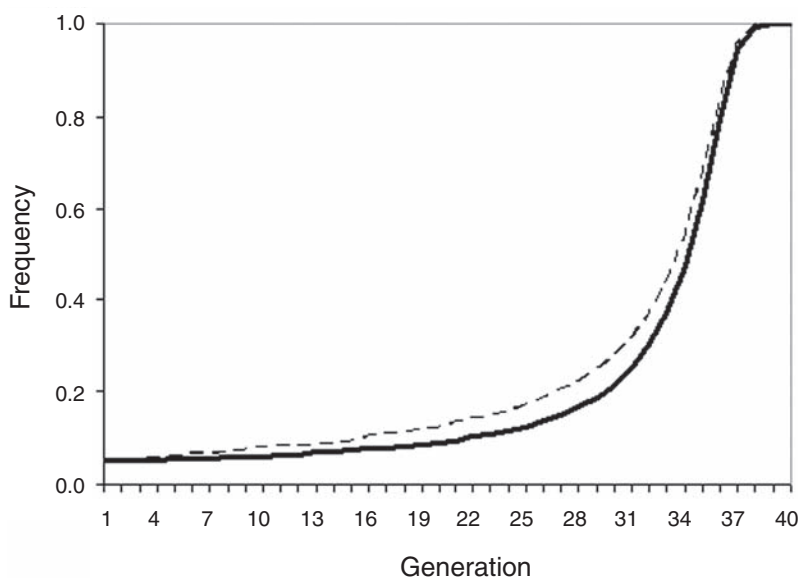


Figure 14.1 *Wolbachia* can drive maternally inherited transgenes into mosquito populations. Solid line = *Wolbachia*. Dotted line = maternally inherited transgene. *Wolbachia* maternal transmission efficiency is 95%, causes 100% CI, and has no fitness cost. Transgene is neutral and is maternally inherited by 100% of progeny. Transgene frequency increases from an initial level of 5% to fixation in approximately 40 generations.

Table 14.1 Species of *Anopheles* That Have Been Assayed for *Wolbachia* Infection and Found Negative

<i>Anopheles gambiae</i> s.s.	<i>Anopheles annularis</i> s.l.
<i>Anopheles funestus</i>	<i>Anopheles culicifacies</i>
<i>Anopheles arabiensis</i>	<i>Anopheles dirus</i> species A & B
<i>Anopheles nili</i>	<i>Anopheles dravidicus</i>
<i>Anopheles pharoensis</i>	<i>Anopheles jamesii</i>
<i>Anopheles moucheti</i>	<i>Anopheles kochi</i>
<i>Anopheles maculipennis</i>	<i>Anopheles maculatus</i>
<i>Anopheles atroparous</i>	<i>Anopheles minimus</i>
<i>Anopheles sacharovi</i>	<i>Anopheles nivipes</i>
<i>Anopheles superpictus</i>	<i>Anopheles pseudowillmori</i>
<i>Anopheles plumbeus</i>	<i>Anopheles sawadwongporni</i>
<i>Anopheles freeborni</i>	<i>Anopheles spendidus</i>
<i>Anopheles barbirostris</i>	<i>Anopheles subpictus</i>
<i>Anopheles peditaeniatus</i>	<i>Anopheles tessellatus</i>
<i>Anopheles hyrcanus</i>	<i>Anopheles vagus</i>
<i>Anopheles aconitis</i>	<i>Anopheles varuna</i>

Source: Kittayapong et al. 2000; Ricci et al. 2002; Rasgon and Scott 2004.

lifespan are theoretically more efficient in reducing disease than other strategies because small changes in the daily survival rate result in large changes in the number of new host infections. A pathogenic *Wolbachia* strain (denoted *popcorn* or *wMelPop*) has been shown to kill adult *Drosophila melanogaster* by over-replication in the central nervous system of the fly. Adult life span of infected flies is approximately one-half that of uninfected flies (Min and Benzer, 1997). Similar results were seen when *wMelPop* was artificially transferred to *D. simulans* (McGraw et al., 2002). If a virulent *popcorn*-like *Wolbachia* strain were transferred into *Anopheles*, it might be possible to use CI to counteract the fitness disadvantages conferred by increased mortality and spread pathogenic symbionts through the population, reducing pathogen transmission and malaria incidence by shortening vector life span (Rasgon et al., 2003).

Wolbachia in *Anopheles*

Wolbachia symbionts have been identified in many mosquito species (Kittayapong et al., 2000; Ricci et al., 2002; Rasgon and Scott, 2004a), and the processes that govern symbiont spread in natural mosquito populations have been examined in detail empirically (Rasgon and Scott, 2003, 2004a) and theoretically (Dobson et al., 2002a; Rasgon et al., 2003; Rasgon and Scott, 2004b), and several different *Wolbachia*-based control strategies have been discussed (Dobson et al., 2002a; Rasgon et al., 2003; Rasgon and Scott, 2003; Sinkins and Godfray, 2004), but no *Wolbachia* infections have ever been identified in any species of *Anopheles* (Kittayapong et al., 2000; Ricci et al., 2002; Rasgon and Scott, 2004a). Over 30 species of *Anopheles* from four continents have been assayed for *Wolbachia* infections with negative results (Table 14.1). Because preexisting natural infections can interact with and alter the behavior of introduced infections (Hoffmann and Turelli, 1997), the naive infection status of

natural *Anopheles gambiae* populations offer a clean slate for *Wolbachia*-based malaria control strategies.

Has Wolbachia ever been associated with Anopheles mosquitoes?

In a study examining the *An. gambiae* salivary gland transcriptome, researchers identified a region on chromosome 3R encoding for several transcripts coding for putatively “*Wolbachia*-like” proteins (e.g., ENSANGP00000026834) associated with cell membrane biogenesis (Arca et al., 2005). This region was flanked by transposons, suggesting a possible transposable element-mediated horizontal transfer event from a past *Wolbachia* infection into the *Anopheles* genome. Horizontal movement of *Wolbachia* DNA into the host nuclear chromosomes is now an established phenomenon (Kondo et al., 2002; Hotopp et al., 2007) and these data, taken at face value, suggest that sometime in the past *Anopheles* may have been infected with *Wolbachia*. The researchers also suggested that the presence of these *Wolbachia*-like transcripts may be responsible for the apparent resistance of *Anopheles* to current infection. However, homologues to these genes are also found in *Aedes aegypti* (e.g., Genbank #EAT45021), indicating that if a horizontal transfer from *Wolbachia* to mosquitoes occurred, it happened before the split of the genera *Anopheles* and *Aedes*. Thus, there is no genomic evidence that *Anopheles* mosquitoes have ever had *Wolbachia* infection.

In vitro studies of Wolbachia–Anopheles interactions

Due to the absence of infection in Anopheline mosquitoes, some have suggested that *Anopheles* mosquitoes may be genetically incapable of sustaining *Wolbachia* infections (Sinkins, 2004; Arca et al., 2005). Our group hypothesized that if there was an intrinsic genetic block to *Wolbachia* infection in *Anopheles gambiae*, we would be unable to infect cultured *Anoph-*

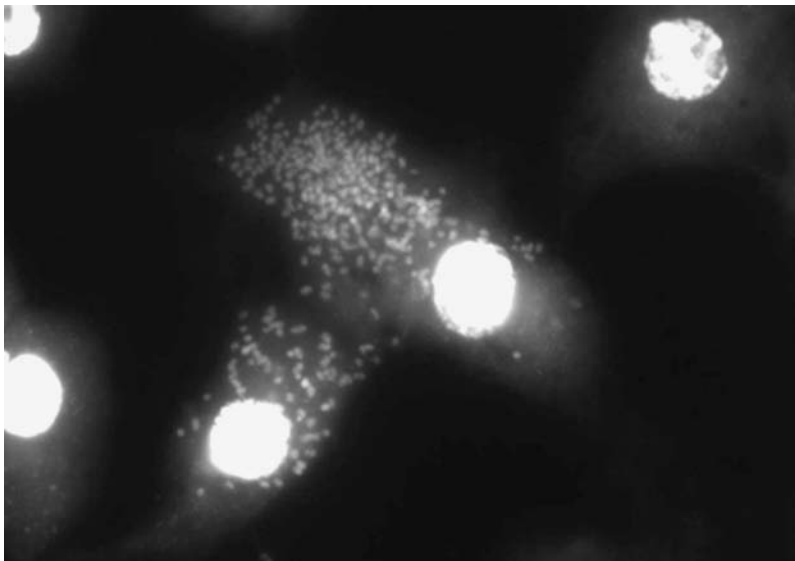


Figure 14.2 *Wolbachia* strain *wRi* in Sua5B cells. Cell nuclei and *Wolbachia* are stained with DAPI and visualized by epifluorescent microscopy.

gambiae cells with the symbiont. Using the modified shell-vial technique (Dobson et al., 2002b), we successfully infected two phylogenetically distinct *Wolbachia* strains—*w*Ri from *Drosophila simulans* (Figure 14.2) and *w*AlbB from *Aedes albopictus* Aa23 cells—into the immune-competent *An. gambiae* cell line Sua5B (Rasgon et al., 2006). Another *An. gambiae* cell line (Moss55) has been infected with the virulent *Wolbachia* strain *w*MelPop (gift of Dr. SL O'Neill, University of Queensland). Some infections have reached very high levels in the cell cultures, where almost 100% of cells are infected at high levels (*w*AlbB in Sua5B and *w*MelPop in Moss55). *w*Ri, however, never reached high levels in Sua5B cells (maximum 30% of cells infected) (Rasgon et al., 2006) and was eventually eliminated from the cell line after approximately 150 passages (Rasgon, unpublished).

Although *in vitro* data do not always translate to results *in vivo*, the cell line data indicate that there is no genetic block to some strains of *Wolbachia* in *Anopheles gambiae* cells, and thus there is no *a priori* reason to suggest that *Anopheles* mosquitoes are refractory to *Wolbachia* infection in general, although certain strains of *Wolbachia* may be more able to colonize *Anopheles* than others. Therefore, with proper technique, and selection of an appropriate *Wolbachia* strain, establishment of *in vivo* *Anopheles* infections may well be feasible.

In vivo *Anopheles* infections with *Wolbachia*

Artificial cross-taxa *Wolbachia* transfections by embryonic microinjection are routine in *Drosophila* (Poinsot et al., 1998; Rousset et al., 1999; McGraw et al., 2002; Riegler et al., 2004; Veneti et al., 2004) and have succeeded in several other insect taxa (Chang and Wade, 1996; Van Meer and Stouthamer, 1999; Sasaki and Ishikawa, 2000; Zabalou et al., 2004). Recently, embryonic microinjection protocols for *Wolbachia* transfection have been developed for the mosquitoes *Aedes albopictus* (Xi et al., 2006) and *Aedes aegypti* (Xi et al., 2005), as well as *Wolbachia* transfer protocols based on injection of symbionts directly into adult *Drosophila* and *Aedes* mosquitoes (Frydman et al., 2006; Ruang-Areerate and Kittayapong, 2006). We are currently using both embryonic and adult injection protocols to transfer *Wolbachia* into *Anopheles gambiae*, and experiments are ongoing.

Conclusions

Although there has been much recent progress toward the goal of developing transgenic *Anopheles* mosquitoes that are refractory to transmission of malaria parasites, there has been little corresponding research toward the development of drive mechanisms. Without a drive mechanism, most transgenic control strategies are doomed to failure. *Wolbachia* has shown considerable promise in both manipulated and natural systems as a viable method for driving genes into populations for disease control. *In vitro* data suggests that the *Anopheles* genetic background is competent to harbor some *Wolbachia* strains and there is thus no *a priori* reason to suspect that the mosquitoes are refractory to infection *in vivo*. *Wolbachia* transfer technologies have been developed for a variety of vector and nonvector insects, and we have every reason to believe that similar techniques can be adapted to *Anopheles* mosquitoes. The successful transfer of *Wolbachia* into *Anopheles* mosquitoes will lay the foundation for the successful deployment of genetically modified *Anopheles* mosquitoes for malaria control.

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Bacterial symbionts in Anopheles spp. and other mosquito vectors

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Introduction

Mosquito-borne diseases (malaria, filariasis, dengue, chikungunya, etc.) represent dramatic health problems, mainly in developing countries where these diseases are endemic. Different integrated approaches have been aimed at both vector control and disease control, the most popular represented by the application of DDT and other insecticides. Insecticide-based strategies, integrated with therapeutic and prophylactic treatment of human hosts, has led to eradication of malaria in several regions around the world. However, control strategies based on chemical insecticides are not always practical due to the economic cost, the characteristics of the region, mosquito breeding sites, etc. In addition, the ecological impact of massive insecticide treatment justifies environmental and human health concerns about their application. Alternative, environment-friendly technologies are thus being explored to overcome such constraints. In this context, “biological control” strategies, based on the use of antagonistic organisms/microorganisms, have attracted a great deal of attention. This approach has already been applied to organic culture, forest protection, and the control of some insect vectors of disease (Vu et al., 2005; Kanzok and Jacobs-Lorena, 2007; Thomas and Read, 2007; Toledo et al., 2007). The microbial communities associated with a given mosquito species offer a cornucopia of biological entities that may be useful for the control of the mosquito itself or the diseases it transmits. This horn of plenty has thus far been investigated only for a few mosquito species, and the overall picture of microbial diversity associated with these insects is still far from being completely drawn. Microorganisms associated with mosquitoes might reduce the host fitness or interfere with vector competence, either as “native” microorganisms or after

appropriate manipulation. In this regard, a bacterium that is associated with almost all families of insects, including mosquitoes, and that has attracted a great deal of attention is *Wolbachia*. This bacterial genus and its potential as a weapon against insect pests and vectors has particularly been treated in the first two volumes of *Insect Symbiosis* (Bourtzis and Miller, 2003, 2006). Here we will focus on other components of the microbial communities associated with mosquitoes, reviewing current knowledge and emphasizing possible applications.

Symbiotic microorganisms offer a route toward the development of novel and environment-friendly strategies for insect pest management. Two approaches have remarkable potential: (1) the elimination of microbial symbionts required by the insect pest/vector for its well-being and survival, and (2) the manipulation of microorganisms with major impacts on insect traits contributing to their pest/vector status like, for example, the capacity to transmit disease. These strategies are obviously attractive for the control of mosquito species of public health concern. Furthermore, the possibility of manipulating bacterial symbionts to produce antipathogen effector molecules has been discussed as a possible tool for malaria control (Riehle et al., 2007).

Despite the fact that the composition of mosquito gut bacterial flora may strongly affect both insect vectorial and immunological competence, only a limited number of studies have thus far been published regarding the structure of the bacterial community present in these insects (e.g., see Pidiyar et al., 2002, 2004; Toure et al., 2000; Demaio et al., 1996). These studies have shown that different mosquito species (belonging to the genera *Anopheles*, *Aedes*, *Culex*, *Psorophora*) harbor common bacterial genera (e.g., *Pseudomonas*, *Staphylococcus*, *Enterobacter*, *Escherichia*), and that several of these bacteria might be manipulated through the exploitation of genetic tools that are already available (vectors, protocols for transformation, etc.). In general, it is reasonable to expect that there is some interaction between the bacteria present in the mosquito gut and the pathogenic microorganisms that the mosquito transmits, particularly in those cases where the pathogen life cycle involves one or more steps in the insect gut, as in the case of malaria parasites, *Plasmodium* spp. Research is obviously needed in this area, for example, on the use of antibiotics to manipulate the composition of the microbiota of the mosquito gut (Toure et al., 2000).

Effects of midgut bacteria on the vector biology

Gonzalez-Ceron and collaborators (2003) investigated the microbiota associated with insectary and field-collected specimens of *Anopheles albimanus* from Mexico; although no bacteria were isolated from laboratory specimens, *Serratia marcescens*, *Enterobacter cloacae*, and *En. amnigenus* were isolated from field-collected mosquitoes. Aseptic specimens of *An. albimanus* were then fed on a blood meal containing these bacteria, together with *Plasmodium vivax*. The number of infected mosquitoes and oocyst density were much lower than in control mosquitoes, thus indicating that the overall midgut bacterial microflora in the vector may influence both *Plasmodium* transmission and periodic variation in malaria incidence. Demaio and collaborators (1996) analyzed the midgut bacterial flora of wild mosquitoes belonging to the species *Aedes triseriatus*, *Culex pipiens*, and *Psorophora columbiae*. The bacterial species that were most frequently isolated were *Se. marcescens*, *Klebsiella ozaenae*, *Pseudomonas aeruginosa*, and *En. agglomerans*. This study emphasized that midgut bacterial counts changed quite dramatically during mosquito development and after the blood meal. Other studies have demonstrated that the midgut of *C. pipiens* can host bacteria of the genera *Bacillus*, *Streptococcus*, *Staphylococcus*, *Salmonella*, and *Shigella*, which influence the digestive processes of the mosquitoes (Fouda, 2001). Furthermore, *Bacillus* and *Staphylo-*

coccus have been reported to be required for the proper completion of the embryonic mosquito development (Fouda, 2001). *Spiroplasma taiwanense*, isolated from *An. sinensis* from Taiwan and then introduced into the breeding water, was shown to reduce the survival of larvae of *Ae. aegypti* (Humphery-Smith et al., 1991). These bacteria, or toxins produced by them, have thus been proposed for use in integrated vector control programs.

Field studies performed in Kenya and Mali analyzed the bacterial midgut contents of mosquitoes belonging to the main African malaria vectors, *An. gambiae* s.l. and *An. funestus*, in order to evaluate the potential relationships between Gram-negative bacteria and *Plasmodium falciparum* sporozoites (Straif et al., 1998). Twenty different genera of bacteria were identified from mosquito midguts and *Pantoea agglomerans* (synonym *En. agglomerans*) was the most common species identified. Even though there was no association between Gram-negative bacteria in the midgut and *P. falciparum* sporozoites in field-collected mosquitoes of either species, *An. funestus* female specimens harboring Gram-positive bacteria were more likely to be infected with sporozoites than mosquitoes harboring Gram-negative bacteria, or with no cultivable bacteria in their midgut.

Bacterial candidates for paratransgenic control of mosquito vectors

Bacterial symbionts could be used as vehicles for expressing foreign genes in mosquitoes. Expression of selected genes could prevent the mosquito from transmitting human and animal pathogens. Obviously, the fundamental prerequisite for an effective protocol of paratransgenesis to control mosquito-borne diseases is the identification of suitable bacteria. The key features that a bacterium should possess for this use are (Beard et al., 2002; Riehle and Jacobs-Lorena, 2005):

- It should be a dominant bacterium within the insect-associated microbiota.
- It should be cultivable in cell-free media.
- It should be readily applicable to genetic transformation.
- It should exhibit stable expression and maintenance of the newly acquired antipathogen function.
- It should have wide distribution in the preadult and adult insect body.
- It should colocalize with the infectious agent in the relevant insect organs (e.g., gut and salivary glands).

The use of molecular identification methods, based on 16S rRNA gene amplification, cloning, and sequencing, in combination with microbiological and biochemical techniques, has made the identification of the microbiota associated with different mosquito species much more rapid. This has allowed the identification of “new” bacterial strains, as in the case of *Aeromonas culicicola* from the midgut of *Culex quinquefasciatus* (Pidiyar et al., 2002).

This multifaceted approach, performed on field caught specimens of *An. gambiae* s.l. and *An. funestus* from West Kenya, led to the identification of 16 midgut-associated bacterial species belonging to different genera, among these *Anaplasma*, *Mycoplasma*, *Nocardia*, and *Janibacter* (Lindh et al., 2005). The genus *Anaplasma* is a “*Wolbachia* sister taxon” and includes several tick-borne species pathogenic to ruminants and humans. The vectorial capacity of mosquito for *Anaplasma* deserves further investigation. The nominal species *Mycoplasma wenyoni* identified in specimens of *An. arabiensis* is closely related to *Mycoplasma suis*, a bacterium mechanically transmitted between pigs by *Ae. aegypti* (Prullage et al., 1993), whereas *Nocardia corynebacterioides* is closely related to *Rhodococcus rhodnii*, a symbiont of the triatomine bug *Rhodnius prolixus*, vector of Chagas’ disease. *R. rhodnii* has already been successfully employed in paratransgenic strategies aimed at controlling the

spread of Chagas' disease, indicating the enormous potential of this approach (Durvasula et al., 1997; Beard et al., 2001, 2002). The *Janibacter* strains detected in *Anopheles arabiensis* represent a new species; the name chosen for this species, *Janibacter anophelis*, emphasizes the close relationship with the mosquito host (Kämpfer et al., 2006b). A γ -proteobacterium has also been shown to be associated with the midgut of *An. arabiensis* and has been named *Thorsellia anophelis* (Kämpfer et al., 2006a). Briones and collaborators (2008) suggested that *T. anophelis* could be usefully employed in paratransgenic control of a malaria vector, because this bacterium appears to be dominant in Kenyan populations of *An. gambiae*. As stated above, dominance is regarded as one of the key features of a symbiont to be employed in paratransgenesis. The dominance of *T. anophelis* in the Kenyan *An. gambiae* population has been assessed through the sequencing of major bands generated in denaturing gradient gel electrophoresis (DGGE) experiments. Further analyses are perhaps required to confirm that this bacterium is dominant in Kenyan *An. gambiae* mosquitoes.

Using bacteria to express mosquito larvicidal protein or anti-Plasmodium molecules

Kampang and collaborators (1999) isolated from *Anopheles dirus* a strain of *Enterobacter amnigenus* able to recolonize the gut of larvae from this species. This capacity looks somehow specific because the bacteria are not able to recolonize in the gut of larvae of *Cx. quinquefasciatus* and *Ae. aegypti*. *En. amnigenus* is able to float in the water for a much longer period than *Bacillus thuringiensis* and *B. sphaericus*, two bacteria that for years have been used in the biological control of mosquitoes and other biting flies (Priest, 1992) and that tend to sink when sprayed in the water, thus requiring many applications of the bacteria to effectively control the mosquito larvae. Since the original experiments of recolonization were performed keeping mosquito larvae without food supplement after feeding with bacteria, further experiments were carried out with larvae that after being fed with bacteria were continuously fed a mosquito larval diet. These experiments indicated that recolonization by *En. amnigenus* was not due to fasting (Kampang et al., 1999).

When the mosquito larvicidal binary toxin of *B. sphaericus* 2297 was expressed in *En. amnigenus*, the effect on *An. dirus* larvae was dramatic (Kampang et al., 2001). Indeed, *En. amnigenus* carrying a recombinant plasmid containing the toxin genes under the control of the native *B. sphaericus* promoter not only expressed an amount of protein comparable to that found in *B. sphaericus* 2297, but also provided around twenty times higher toxicity toward second instar larvae of *An. dirus* if compared to *B. sphaericus* 2297. Even though the species-specific pattern of recolonization of *E. amnigenus* is a limiting factor to its wide application, it still remains a promising candidate for field applications of mosquito control.

Riehle and collaborators (2007) genetically engineered *Escherichia coli* to display anti-*Plasmodium* effector molecules. In particular, two molecules that already proved to be particularly attractive were used in this set of experiments:

1. SM1, a small dodecapeptide able to interfere with a binding protein of the lumen of the mosquito midgut, needed for *Plasmodium* invasion, and, as a consequence, interfering with parasite development (Ghosh et al., 2001)
2. PLA2, a snake venom phospholipase, which also blocks *Plasmodium* development in the mosquito midgut by the inhibition of the association between ookinetes and midgut surface (Zieler et al., 2001)

E. coli bacteria expressing both molecules, and supplied with food to mosquitoes 24 hours prior an infective blood meal, inhibited the parasite development quite significantly (41% and 23%, respectively). Nevertheless, *E. coli* survived poorly in mosquitoes; therefore, *Enterobacter agglomerans* was isolated from mosquito midguts and selected for midgut survival by the means of serial passages in mosquitoes: after four passages survivorship increased from 2 days to 2 weeks. Due to the fact that *En. agglomerans* is widespread and nonpathogenic, it is to be regarded as a good candidate for the development of paratransgenic protocols aimed at the control of mosquito vectors.

A “promising” acetic acid bacterium for paratransgenic control of malaria vectors

Recently, we described an α -proteobacterium of the genus *Asaia* that is stably associated with different *Anopheles* species (Favia et al., 2007). *Asaia* was found to massively colonize the midgut and the male reproductive system of adult *An. stephensi*. Indeed, *in situ* hybridization and transmission electron microscopy (TEM) revealed that the midgut of adult *An. stephensi* harbors dense clusters of bacteria that appear extremely uniform at the ultrastructural level and are embedded within a thick slime matrix (Figure 15.1A). Slime matrix is typically produced by the Acetobacteraceae, the bacterial family to which *Asaia* belongs. At higher magnification, the Gram-negative architecture of *Asaia* is evident, as well as the filamentous appearance of the nucleoid region surrounded by an electron dense cytoplasm (Figure 15.1B). In addition, *in situ* hybridization and TEM revealed that *Asaia* also localizes within the male gonoduct, forming large microcolonies. In particular, TEM reveals the presence of plugs of bacterial cells within the male deferent duct, presenting the same overall architecture of bacteria in the gut and embedded within a similar extracellular matrix (Figures 15.2A and B). These observations suggest that *Asaia* might follow a paternal route of transmission to the offspring, as well as a venereal route for horizontal transmission.

Asaia thus appears to be a dominant bacterium within the body of *An. stephensi*, one of the major malaria vectors in Asia. Molecular and microbial analysis also revealed the presence of *Asaia* in field collected samples of *An. maculipennis*, a European mosquito vector, and *An. gambiae*, the main African malaria vector (Favia et al., 2007).

Asaia shows a series of characteristics that make it one of the best available candidates for development of paratransgenesis-based strategies for the control of malaria. Indeed, *Asaia* is characterized by (1) dominance within the mosquito-associated microflora, as revealed by clone prevalence in 16S rRNA gene libraries and quantitative real-time polymerase chain reaction (qRT-PCR); (2) cultivability in cell-free media; (3) transformability with foreign DNA; (4) wide distribution in the larva and adult mosquito body, as shown by TEM and *in situ* hybridization; (5) colocalization with malaria parasite (in the midgut and salivary glands), and a further localization in the reproductive organs of both sexes.

By the use of a bacterial strain modified to express the green fluorescent protein (GFP) and added to sugar or blood meal, we were able to demonstrate the ability of *Asaia* to quickly colonize the gut (Figure 15.3), salivary glands, and male reproductive organs of mosquitoes. Through mating experiments we also demonstrated the high transmission potential of the symbiont from mosquito parents to the progeny, likely through different mechanisms. *Asaia* is also capable of horizontal infection through an oral route during feeding both in preadult and adult stages and through a venereal pattern during mating in adults. This capacity of *Asaia* of being both vertically and horizontally transmitted in mosquitoes and its capacity for surviving and reproducing in the environment highlight

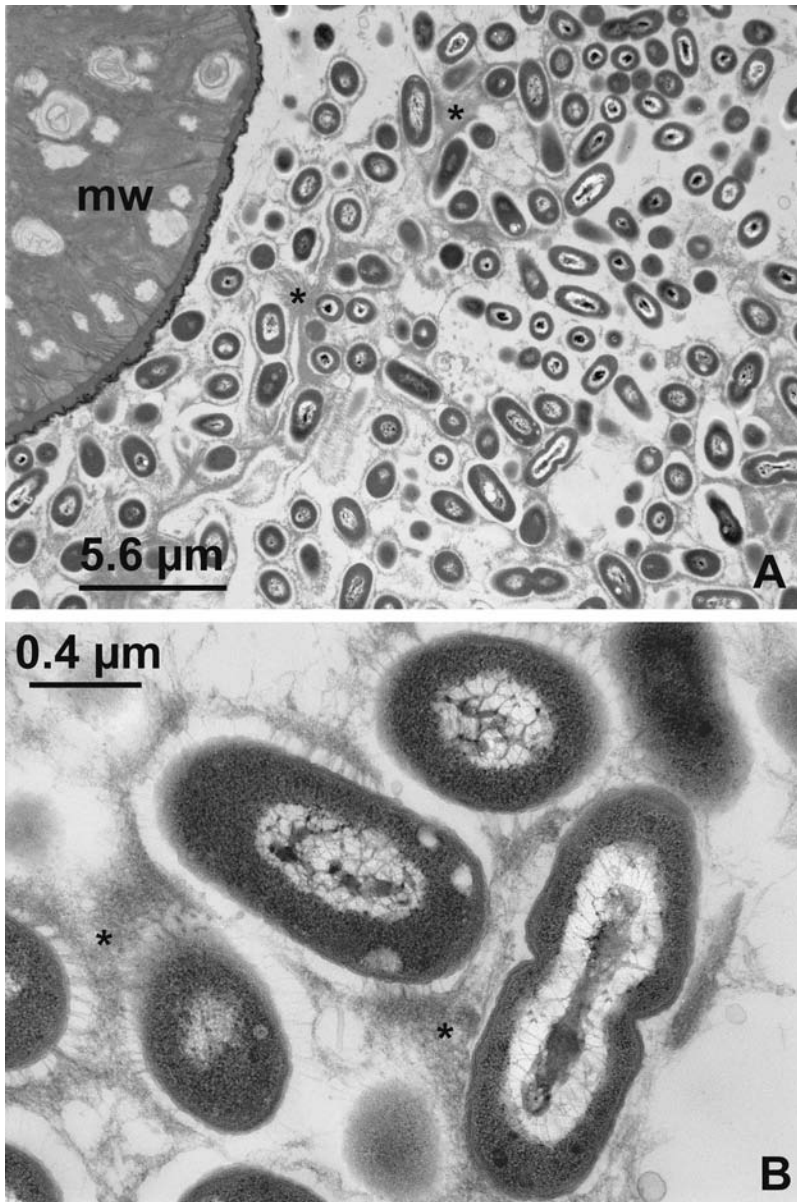


Figure 15.1 TEM micrographs of the midgut of adult females of *Anopheles stephensi*, showing: (A) the lumen full of *Asaia* (asterisks = extracellular slime matrix; mw = midgut wall); (B) details of *Asaia* symbionts, characterized by the presence of an extracellular slime matrix (asterisks), an electron-dense cytoplasm, and a filamentous nucleoid region.

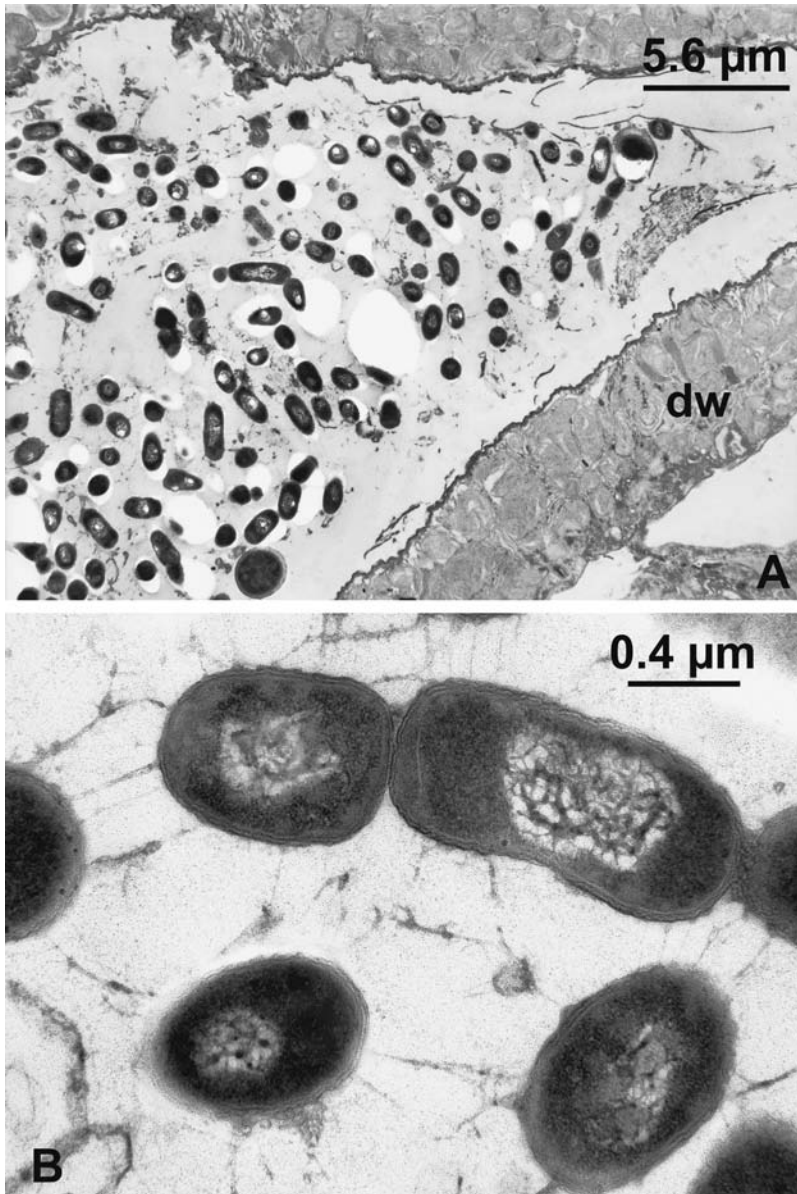


Figure 15.2 TEM micrographs of the adult male deferent of *Anopheles stephensi*, showing (A) a plug of *Asaia* symbionts (dw = deferent wall) and (B) details at higher magnification, illustrating the characteristic architecture of *Asaia*.

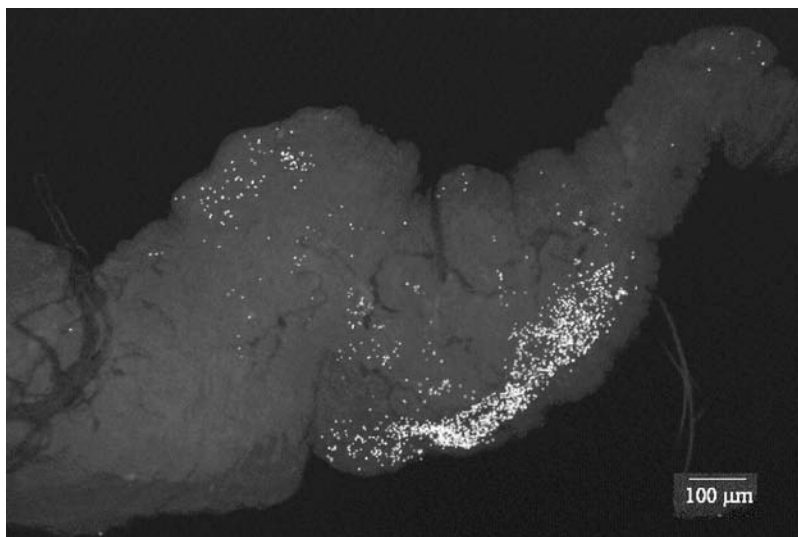


Figure 15.3 (Color figure follows p. 238.) The gut of a female *Anopheles stephensi* mosquito, colonized by transformed bacteria of the genus *Asaia* that express the green fluorescent protein (GFP). A massive colonization is located at the midgut level.

the potential of this bacterium for its use in the control of insect pests and insect vector capacities. It is interesting to note that *Asaia* has also been found in other insects, including hemipteran vectors of plant diseases (Marzorati et al., 2006; see also Alma et al. in this volume). This bacterium could thus represent a multipurpose weapon for the control of vector borne diseases, both in the medical and veterinary fields, as well as in the area of plant diseases.

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Symbiotic microorganisms in leafhopper and planthopper vectors of phytoplasmas in grapevine

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Introduction

The most important phytopathogenic agents causing diseases of economic concern to grapevine—often of an epidemic nature—are viruses, bacteria, and phytoplasmas. Such agents are transmitted in nature by animal vectors: viruses by nematodes and mealybugs (pseudococcids and coccids); bacteria and phytoplasmas, the latter with an always growing phytopathological interest, by leafhoppers (cixiids and cicadellids). All the diseases caused by phytoplasmas are described by the term *grapevine yellows*. The yellows are spread in the main grapevine-growing areas of the world, such as central and southern Europe, the Middle East, North and South Africa, North and South America, and Australia (Bianco et al., 1996; Batlle et al., 1997; Boudon-Padieu, 2000; Angelini et al., 2001; Bianco et al., 2001; Boudon-Padieu, 2003).

Flavescente dorée (FD), a disease that forces countries to place affected areas in quarantine, frequently affects vineyards in southern Europe and provokes a heavy economic impact on grapevine production. The FD phytoplasma (elm yellows) is transmitted in an epidemic way from grapevine to grapevine by the leafhopper of Nearctic origin, *Scaphoideus titanus*. This leafhopper is strictly ampelophagous, as it is able to accomplish its life

history only on *Vitis* spp. Besides on the European grapevine, it is reported also on different species of American grapevine (Beanland et al., 2006). Another emerging yellows, which is more and more worrying for the damage caused in vineyards of Mediterranean Europe, is bois noir (BN). The phytoplasma responsible for BN (Stolbur group) is transmitted by the planthopper *Hyalesthes obsoletus*, a polyphagous species living on several wild and cultivated herbaceous dicotyledons, often hosting Stolbur phytoplasmas, sometimes with no clear symptoms. The phytoplasma is transmitted occasionally by *H. obsoletus* to grapevine that is considered the end host of the pathogen (Lessio et al., 2007).

The control of phytoplasma-borne diseases is achieved through the use of insecticides against the vectors and the removal of diseased plants. FD control is obligatory in some European countries. Such measures applied in whole grapevine-growing districts have severe effects on nontarget invertebrates. Alternative and environmental-friendly control strategies are thus strongly needed. An example is symbiotic control, an approach recently proposed for the control of Pierce's disease (Bextine et al., 2004, 2005; Miller et al., 2006).

Very few investigations have been made on the symbiotic microbiota associated with the leafhopper and planthopper vectors of phytoplasmas in grapevine. This chapter is intended to give an overview on the actual knowledge on the microbial symbionts that have been characterized in hemipteran vectors of bacterial diseases in grapevine, with a particular focus on insect vectors of phytoplasma-borne diseases. We first give an overview on the phytoplasmas and their leafhopper and planthopper vectors, and hence we summarize the knowledge on the vector microbial symbionts that have been studied before the end of 2007.

Phytoplasma vectors

Phytoplasmas belong to the class *Mollicutes* and are phytopathogenic agents that are non-culturable *in vitro*. They are obliged parasites, localized in the phloem tubes and the companion cells. Thanks to their small size and the lack of a cell wall they can move from one cell to the other through the pores of the sieve plates. They are associated with over 1,000 diseases of wild and cultivated plants all over the World (Lee et al., 2000). The diseases caused by phytoplasmas, creating serious damage and often killing the plant, are so devastating that in some cases they cancel the yield of the plants in those regions where they are endemic or into which they are introduced. Among the most important diseases caused by phytoplasmas, we especially remember the palm lethal yellows, the Australian grapevine yellows, the alfalfa virescence and witches' broom, and the aster yellows in different vegetable crops. Besides grapevine yellows (FD and BN), the phytoplasma-caused diseases with major economic impact in Europe, there are the apple witches' broom, the stone fruit tree yellows, the pear decline, and the tomato stolbur.

Phytoplasma genomes are smaller than 1 Mb (0.7 and 0.86 Mb for the aster and the onion yellows phytoplasmas, respectively) and have been regarded as the result of a process of reductive evolution (Oshima et al., 2004; Bai et al., 2006). Phytoplasmas are intrinsically unculturable outside a host because they lack several functions that are generally recognized as essential for free-living prokaryotes. These include, among others, the pentose phosphate cycle, several essential transporters and pathways, such as the phosphoenolpyruvate:sugar phosphotransferase systems for importing sugars essential for glycolysis, and ATP-synthase subunits. Such evidence indicates that they are relatively inefficient to generate ATP. Their genomes are rich in small repeats that are organized in large clusters of potential mobile units (PMU). These have been proposed to be involved in recombination mechanisms for the creation of the variability needed for adjusting to the diverse

environments of the plant and the insect hosts (Bai et al., 2006). The general features of the genomes suggest that phytoplasmas are strictly dependent on the eukaryotic hosts, the plant, and the insect vector.

The insect vectors of phytoplasmas belong to the order Hemiptera (= Rhynchotha), suborder Homoptera, and to the families Cixiidae, Delphacidae, Derbidae, Cercopidae, and Cicadellidae, section Auchenorrhyncha, and to the family Psyllidae, section Sternorrhyncha. In the section Auchenorrhyncha the most investigated family is that of Cicadellidae, with over 70 vector species belonging to the subfamilies Agalliinae, Aphrodinae, Cicadellinae, Coelidiinae, Deltocephalinae, Iassinae, Idiocerinae, Macropsinae, Scarinae (= Gyponinae), and Typhlocybinae. In the single subfamily Deltocephalinae over 50 species are known to be involved in the transmission of phytoplasmas responsible for plant diseases, in some cases of economic importance to wild and cultivated herbaceous, shrubby, and arboreal mono- and dicotyledons. Among the Sternorrhyncha, the family Psyllidae, with different species belonging to the genus *Cacopsylla*, is the most investigated for the link with cultivated pome fruit trees and stone fruit trees and for the crucial role in transmitting phytoplasmas belonging to the apple proliferation group, associated with the most important diseases of fruit trees in temperate regions (Weintraub and Beanland, 2006).

Several plant-sucking insects, such as most of Auchenorrhyncha and numerous Sternorrhyncha, provided with piercing and sucking mouth parts, feed by inserting their stylets into the phloem (Figure 16.1). The phloem is a complex tissue both anatomically and physiologically and is made of different elements, among which are the sieve cells, the companion cells, the phloem parenchyma, and the phloem fibers. Whereas the Sternorrhyncha are able to reach the vessels conducting phloem sap with an extreme precision, the Auchenorrhyncha prove to establish different relationships with the phloem tissue. The companion cells likely represent the main objective of the phloem-sucking leafhoppers. During their feeding, they suck cell contents and inject some saliva that, besides the chemical-enzymatic action, may become the means to inoculate phytoplasmas. The insects, while feeding on an infected plant, may acquire the phytoplasmas and, once they have become infective, transmit them subsequently to healthy plants, also of different species from those on which the acquisition occurred. The transmission process, of a persistent propagative kind, is characterized by three moments, or “phases,” that are distinct but closely interdependent: (1) acquisition access period, (2) latent period, and (3) inoculation. After the acquisition, lasting variably from hours to days, there is a latency period of 2–4 weeks. During this phase the phytoplasmas, introduced into the gut with the phloem sap, reach different organs, including salivary glands, and multiply.

The trophic relation with the plants, to which the infective individuals may transmit the phytoplasmas, can be of an obligatory, facultative, or occasional kind. The occasional species, with adults able to feed on several herbaceous, shrubby, and arboreal plants—many of which are not cultivated and thus unpredictable—in particular ecological conditions may become extremely dangerous. The ability to acquire phytoplasmas from infected plants is higher in the juvenile stages (nymphs) than in the adult stage. The neanids (the first juvenile stages of heterometabolous insects) are not always able to acquire phytoplasmas, because of the reduced length of mouth stylets that do not let them easily reach the phloem tubes. The retention of infectivity is not affected by molts and the insect remains infected for all its life. In some species the females are more efficient than the males in transmitting the phytoplasmas. The different behavior (females more static, males more mobile) is probably the cause of the different transmission capacity. Also, the flight activity and the consequent dispersal ability of the different species condition the transmission

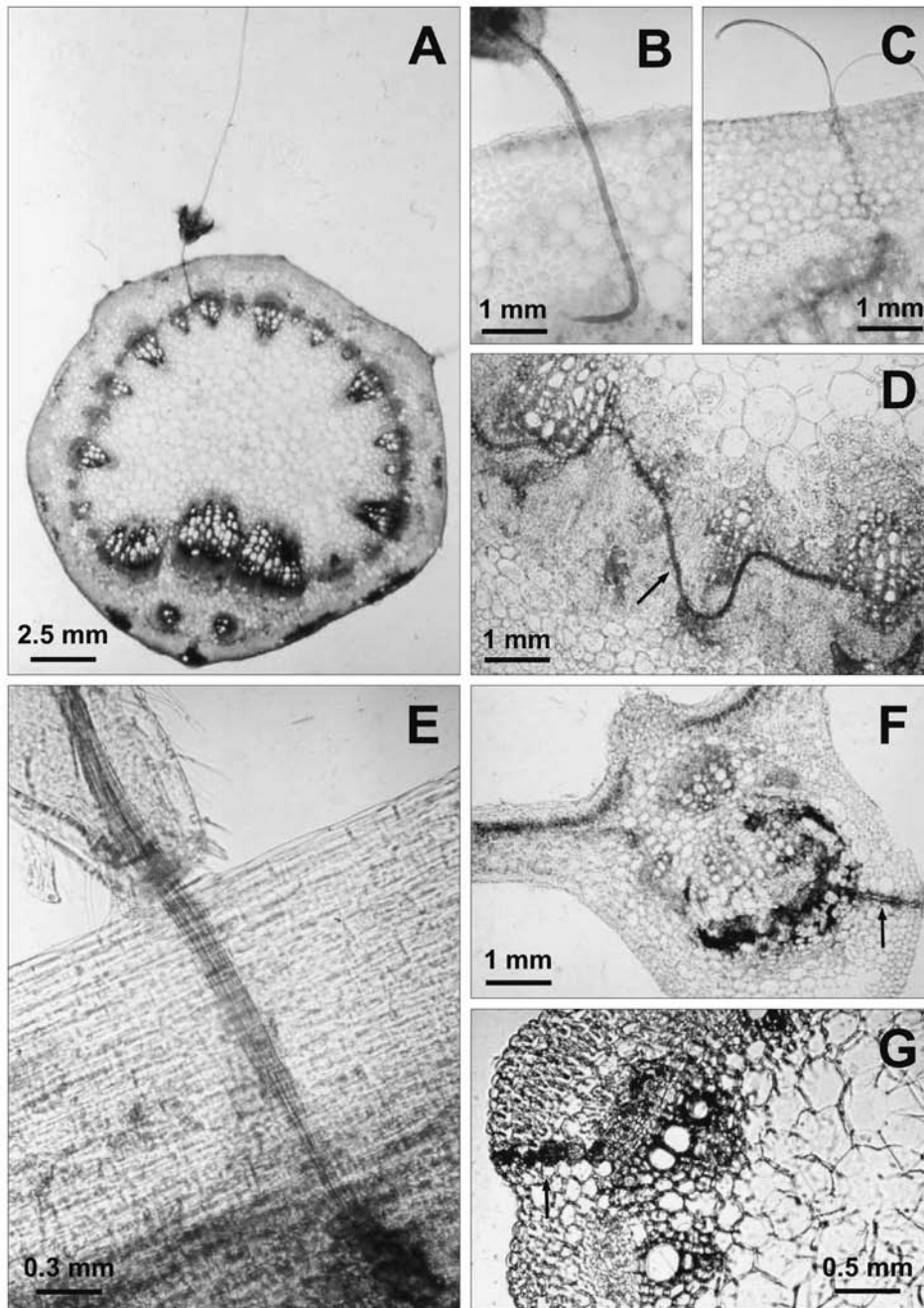


Figure 16.1 (Color figure follows p. 238.) Details of stylets of phloem sucking insects and grapevine leaf tissues during biting. (A–D) *Peryceria purchasi* stylet penetrating grapevine tissues. (A–C) The pictures show the plasticity of the stylet that is inserted between cells in the parenchymatic tissue to reach the phloem cells. (D) Details of the stylet track (arrow) within the leaf tissue showing that the stylet of *P. purchasi* has explored different phloem tubes. (E–G) Biting of grapevine tissues by *S. titanus*. (E) The stylet of *S. titanus* penetrating the leaf tissues. (F and G) Tracks (arrows) left by the stylet of *S. titanus* in the vein of grapevine leaves.

efficiency and characterize the epidemiology of the different phytoplasma diseases (Marzachi et al., 2004).

Recently Weintraub and Beanland (2006) have reviewed the vectors of phytoplasmas. They pointed out that Hemiptera collectively possesses several characteristics that make its members efficient vectors of phytoplasmas: (1) they are hemimetabolous, with nymphs and adults feeding similarly and in the same physical location, and both able to transmit phytoplasmas; (2) they feed specifically and selectively on certain plant tissues, which makes them efficient vectors of pathogens residing in those tissues; (3) they have a propagative and persistent relationship with phytoplasmas; (4) they have obligate symbiotic prokaryotes that are passed to the offspring by transovarial transmission. The same mechanism that allows the transovarial transmission of the symbionts likely provides a preadaptation for the transmission of phytoplasmas. For many years it was considered that phytoplasmas were not transmitted vertically to the progeny of infected insects, although an aster yellows phytoplasma has been reported in eggs, nymphs, and adults of the experimental vector *S. titanus* reared on healthy plants (Alma et al., 1997). Subsequently, the possibility of transovarial transmission was confirmed with the leafhoppers *Hishimonoides sellatififormis*, a vector of the mulberry dwarf phytoplasma (Kawakita et al., 2000), *Matsumuratettix hiroglyphicus*, a vector of the sugarcane white leaf phytoplasma (Hanboonsong et al., 2002), and the psyllid *Cacopsylla pruni*, a vector of the European stone fruit yellows phytoplasma (Tedeschi et al., 2006). Mitsuhashi and collaborators (2002) found *Wolbachia* in *H. sellatififormis* coexisting in all tissues with the phytoplasmas, suggesting that this other prokaryote may have mediated infection by the phytoplasma. It still remains to prove the role of transovarial transmission in the epidemiology of phytoplasma diseases. The fact that the insect is not only a vector, but also a reservoir of the phytoplasma, besides the plant reservoirs, has important implications for disease management. In addition, the possibility of transovarial passage adds to the difficulty of disease control.

Leafhopper and planthopper vectors of phytoplasmas in grapevine

Grapevine yellows associated with phytoplasmas are among the main problems of viticulture worldwide. Currently, three types of yellows are present in Europe, and are caused by phytoplasmas belonging to two ribosomal groups: (a) flavescence dorée (FD) (subgroups –C and –D) and Palatinate grapevine yellows (PGY) (subgroup –A), belonging to the elm yellows group 16SrV, and (b) bois noir (BN) belonging to the stolbur group (16SrXII-A). The grapevines affected by different phylogenetic groups of phytoplasmas react with identical responses. Therefore, the examination of the symptoms on its own does not permit recognition of the different diseases, and it is necessary to turn to molecular diagnostic techniques. Different symptom expressions are, instead, conditioned by grapevine genetics; in fact, one can see very diverse behaviors and reactions in grape varieties and in rootstocks, the latter often being asymptomatic. The most typical symptoms are leaf yellowing (white grape varieties) or reddening (black grape varieties), thickening and downward rolling of the leaf blade, poor lignification of canes, and partial or total desiccation of grape bunches. In some very sensitive varieties the infection remains for some years until it causes the death of the plant.

The leafhopper species presently known to transmit the phytoplasma agents of grapevine diseases belong to the families Cixiidae and Cicadellidae, and are listed in Table 16.1. For each species the systematic position, the chorology, the number of generations, the overwintering mode, and the diseases caused are given. PGY is currently the least worrying and most localized disease, recorded in the grapevine-growing region of Palatinate

Table 16.1 Vectors of Grapevine Phytoplasmas in Europe

Vector	Chorology	Generations	Overwintering	Disease
Cixiidae				
<i>Hyalesthes obsoletus</i>	Palaeartic	1	nymph	BN ^a
Cicadellidae				
Macropsinae				
<i>Oncopsis alni</i>	Palaeartic	1	egg	PGY ^b
Deltocephalinae				
<i>Scaphoideus titanus</i>	Nearctic	1	egg	FD ^c

^a BN = bois noir.^b PGY = palatinate grape yellows.^c FD = flavescence dorée.

in Germany. It is transmitted by the leafhopper *Oncopsis alni* (Maixner et al., 2000). This leafhopper dwells on broadleaves of the genus *Alnus*, and in particular on the species *A. glutinosa*, where it accomplishes its whole life history and transmits the alder yellows caused by phytoplasmas of the same phylogenetic group of FD (16SrV, elm yellows) (Arnau et al., 2007). In environments with the presence of alders and grapevines, from spring to the beginning of summer, the leafhopper adults, by feeding occasionally on grapevine, transmit the phytoplasma that causes the disease known as PGY. The capacity of *S. titanus* to transmit different strains of phytoplasmas closely related to FD is still to be investigated, as well as the possible role of alder as an FD reservoir.

Scaphoideus titanus and *flavescence dorée*

The first disease observed and reported internationally was FD, a kind of yellows still today mostly feared for its rapidity to spread and for the economic damage it can cause. This disease appeared in the middle of the 1950s with showy leaf yellowings and vegetative impairment in vineyards of southwestern France. Even if over the years this disease spread to other countries of western and eastern Europe, the grapevine-growing areas of South France and North Italy remained the most affected ones, with serious production losses.

The leafhopper vector of FD, *S. titanus*, originating from North America, is monophagous on grapevine, and was found in the Palaeartic area for the first time in the 1960s, in southern France. Presently this leafhopper is spread, with a nonuniform distribution, from Portugal to Hungary. Besides the natural colonization of new territories, there is the real possibility that, by trading nursery material (rootstocks, cuttings), humans increase the diffusion of *S. titanus* to other parts of the world where grapevine is grown. Comparing the climograms (rainfall, mean temperature) of North American regions, where the leafhopper was found, based on the data published by Barnett (1977), with some of the most important grapevine-growing areas worldwide, one can see how the different zones in Europe, South Africa, South America, Asia, Australia, and New Zealand could be potentially colonized by *S. titanus*. Such a hypothetical risk, in order to occur practically, needs to reach proper environmental conditions for the completion of the insect's life history, in particular, a winter season with a cold period sufficiently long for the overwintering eggs

to hatch in the following spring. Recently the risk and the possibility have been pointed out that the vector also establishes itself in different grapevine-growing regions of China (Ge and Wen, 2006).

S. titanus accomplishes one generation a year and overwinters in the egg stage laid in two-year-old bark. Every female lays over 20 eggs. The egg hatching starts in the second half of May and continues until after the first ten days of July. The juveniles stay on the lower page of the basal leaves of the shoots close to the canes and to the trunk in which the overwintering eggs were laid. The spatial distribution of *S. titanus* juveniles in the vineyard is of an aggregated kind, with more or less high concentrations on few plants. The knowledge of this particular behavior permitted the development of an efficient monitoring sampling method useful for research and technical assistance (Lessio and Alma, 2006). The adults are active for the whole summer until the beginning of autumn; they have a flight activity mostly crepuscular and tend to fly a little above the grapevine canopy, preferring cultivated vineyards, and are influenced by the planting and canopy density (Lessio and Alma, 2004a, 2004b).

Juveniles accomplish the phytoplasma acquisition mostly from the third instar. After a latency period of about one month, the adults are able to transmit the disease to healthy grapevine plants. The different cultivars, having different sensitivity toward the disease, may influence the acquisition efficiency (Bressan et al., 2005b). The transmission possibility increases at the end of summer, from late August to mid-September, because of the increase of the titer concentration of phytoplasmas in the plant and of the adult density in the field (Lessio et al., 2003; Bressan et al., 2006b). The acquisition of the FD phytoplasma causes in *S. titanus* a lower survival in both sexes and a lower fecundity in females, pointing out a recent coevolution between FD and its specific Nearctic vector (Bressan et al., 2005a). In the laboratory, by using the injection method to directly introduce the pathogen into the hemocoel of potential vectors, it was proved that, besides *S. titanus*, three more leafhopper species are potential vectors of FD in Europe. The three species resulted to be able to acquire FD from infected broad beans and to transmit it to healthy plants, opening new hypotheses on the origin and diffusion of FD in the field (Bressan et al., 2006a). Investigations in the field in different grapevine-growing agro-ecosystems also permitted the detection of the FD phytoplasma in the wild plant *Clematis vitalba* and in the leafhopper *Dictyophara europea*, and to hypothesize for this plant the role of natural reservoir for the FD phytoplasma and for this leafhopper the role of potential vector (Filippin et al., 2007).

Hyalesthes obsoletus and *bois noir*

Bois noir phytoplasmosis was indicated since the beginning, for some infectivity features (nonepidemic), as a grapevine disease close but not identical to FD, because it is not transmissible by means of the leafhopper *S. titanus*. Current knowledge confirms that BN is caused by a phytoplasma nonspecific for grapevine, transmitted by not strictly ampelophagous vector(s). Such an epidemiological situation, distinctly different from FD, reflects on the life cycle of the etiological agent of BN, involving different host plants, besides grapevine, and presumably different vectors, besides *H. obsoletus*, which is presently the only ascertained vector.

BN is a long-known typical grapevine yellows in France and is widespread in different grapevine-growing areas of central and Mediterranean Europe, where it was called different names (Vergilbungskrankheit [VK] in Germany, and Legno Nero [LN] in Italy), and in the Middle East (Lessio et al., 2007). In the last few years this disease has constantly spread and stirred more and more concern for production and control. The stolbur phytoplasma

infects a high number of wild and cultivated plants, in particular vegetables. The disease was first described in central-eastern Europe as epidemic in Solanaceae, such as pepper, tomato, and eggplant. Among wild plants this phytoplasma was found in arboreal and herbaceous hosts, many of which are commonly found in the vineyard agro-ecosystem.

H. obsoletus is widespread in Europe, the Middle East, Asia Minor, and Afghanistan. *H. obsoletus* is a polyphagous and heterotopous species that accomplishes, in Europe, one generation per year and overwinters as a juvenile, mostly in the stage of third instar nymph, on the roots of different wild herbaceous plants, among which the most common ones are nettle (*Urtica dioica*) and convolvulus (*Convolvulus arvensis*), at a depth of about 100–150 mm (Alma et al., 1988). The adults are active in summer and feed occasionally on several herbaceous and shrubby broadleaf plants (Alma et al., 1988; Sforza et al., 1999; Sharon et al., 2005). *H. obsoletus* may be found more frequently in the grapevine-growing areas where its herbaceous host plants, which are indispensable for egg laying and the development of juveniles, are spread. In these environments the adults feed on grapevine occasionally and for very short times, but enough, however, to inoculate BN, as it was proved also by means of laboratory trials (Lessio et al., 2007).

In spite of such evidence, the widespread and varying incidence of this ampelopathy, also in grapevine-growing areas where *H. obsoletus* was not found, and also the peculiar life cycle of the vector, with underground juveniles, lead to the hypothesis of the involvement of other wild plant hosts as natural infection sources of BN and of different vectors. Concerning the role of other leafhoppers or planthoppers, commonly spread in the vineyard agro-ecosystem, serological and molecular investigations detected the stolbur phytoplasma in many other species and a relevant interest assumes in particular its detection in species of the family Cixiidae, such as *Reptalus panzeri* (Palermo et al., 2004), *Reptalus quinquecostatus*, *Hyaletthes luteipes* (Trivellone et al., 2005), and *Pentastiridius beieri* (Gatineau et al., 2001). For all of them the role in checked transmission trials still has to be proved.

A diverse microbiota inhabits S. titanus and H. obsoletus

At first glimpse of the microbial diversity associated with *S. titanus* and *H. obsoletus*, a complex microbial community can be discerned. Having as a purpose a preliminary screening to estimate the whole microbial diversity in arthropod vectors, the use of microbial community fingerprinting methods represents an easy and straightforward approach (Marzorati et al., 2006). There are several methods that can be used, in which conserved genes working as molecular chronometers like the rRNA genes are amplified by PCR and separated on the basis of their length or sequence polymorphisms. Among the others, easy-to-use techniques are LH-PCR (length heterogeneity PCR; [Brusetti et al., 2006]), T-RFLP (terminal-restriction fragment length polymorphisms; [Donovan et al., 2004]), or SSCP (single strand conformation polymorphisms; [Mohr and Tebbe, 2006]). In an LH-PCR survey to study the bacterial microbiota associated to *S. titanus*, Marzorati et al. (2006), found that some LH-PCR peaks presumably attributable to different bacterial species could be discerned in field-recovered leafhopper individuals. With LH-PCR, different bacteria are discriminated by sequence length differences in portions of the 16S rRNA genes that include two variable sequence regions of the gene (Brusetti et al., 2006). Fragments with different lengths are recognized in the electropherogram by different peaks. In Figure 16.2A is shown, as an example, a typical LH-PCR electropherogram obtained from PCR amplification of a whole individual DNA of *H. obsoletus*. Several peaks can be noted, indicating that the bacterial biota associated to this insect vector is rather heterogeneous.

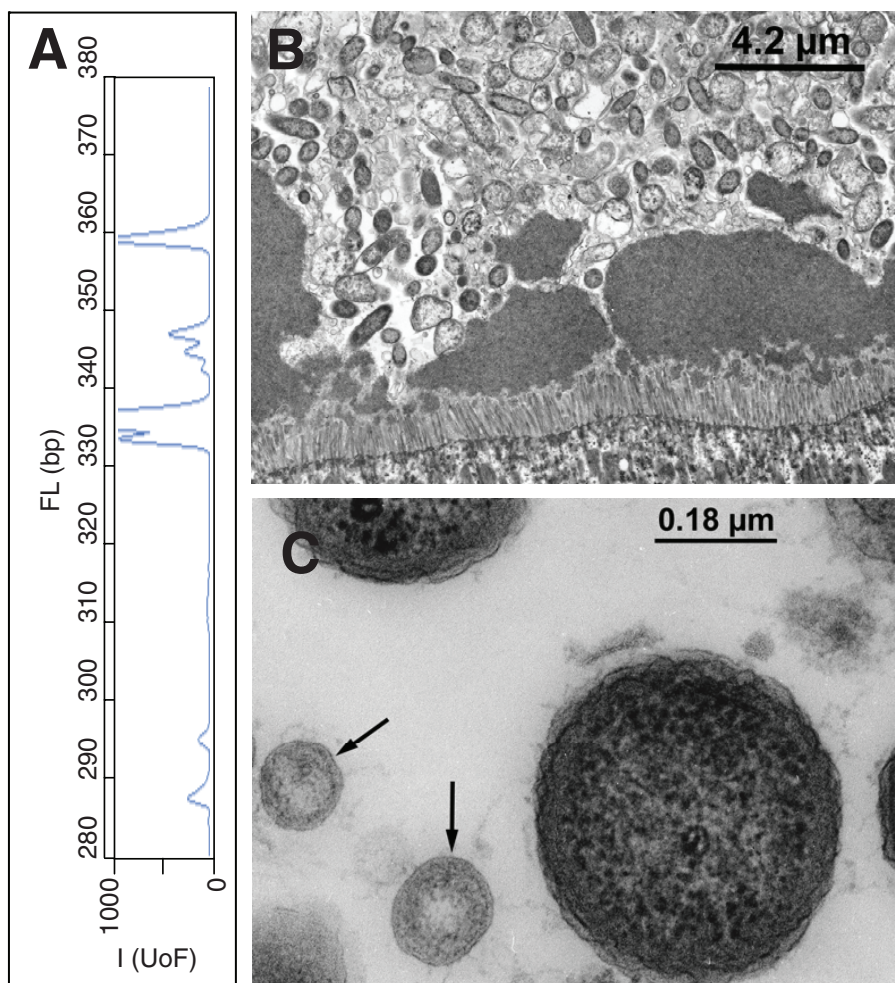


Figure 16.2 Diversity of bacteria associated to *S. titanus* and *H. obsoletus*. (A) 16S rRNA gene LH-PCR electropherogram showing the bacterial diversity associated with a whole individual of *H. obsoletus*. Each peak should represent at least a bacterial species. (B) Several bacterial morphologies can be observed in the gut of *S. titanus* observed by TEM. (C) Two phytoplasma cells (arrows) close to two larger Gram-negative bacterial cells in the midgut of an adult female of *S. titanus*.

Such heterogeneity in Hemiptera was confirmed by transmission electron microscopy (TEM) ultrastructure analysis. For example, in the midgut of *S. titanus* several bacterial morphologies with different ultrastructural characteristics can be observed (Figure 16.2B; Sacchi et al., 2008). This observation agrees with preliminary work showing, by LH-PCR, 16S rRNA gene PCR-DGGE (denaturing gradient gel electrophoresis), and fragment sequencing, which in the leafhopper inhabits Bacteroidetes of the genus *Cardinium* and *Chriseobacterium*, α -Proteobacteria of the genus *Asaia*, and γ -Proteobacteria of the genus *Stenotrophomonas* (Marzorati et al., 2006).

Sacchi et al. (2008) showed that it is possible to trace the FD phytoplasma in the gut of *S. titanus* by TEM. Cells with the typical phytoplasma morphology were observed in the midgut of both adults and nymphs always intermixed with Gram-negative bacteria

(Figure 16.2C; Sacchi et al., 2008). The physical proximity between gut symbiotic flora and phytoplasmas in the midgut opens the perspective that a paratransgenic approach exploiting those symbionts for controlling phytoplasma proliferation is in theory possible. Recently we found that α -Proteobacteria of the genus *Asaia* are the dominant symbionts in the malaria vector *Anopheles stephensi* and have been proposed as potential candidates for controlling the transmission of the malaria parasite in a symbiotic control approach (Favia et al., 2007). It would be interesting to more carefully look at this kind of symbiont in *S. titanus* and *H. obsoletus* by evaluating their prevalence and localization in the body and the role for the host, information that is important for the design of a symbiotic control approach.

Inherited symbionts in grapevine-feeding leafhoppers and planthoppers

Among symbionts that revealed a major interest in recent years are those able to spread into insect host populations by manipulating host reproduction. These microorganisms are intracellular symbionts able to enter female germ line cells and to be directly transmitted to the progeny. The most known models of these manipulators of host reproduction are the α -Proteobacterium *Wolbachia* and the Bacteroidetes *Cardinium*. In particular for *Wolbachia* a vast literature has been produced in the last few years. In several arthropods these bacteria are able to manipulate host reproduction by determining cytoplasmic incompatibility (CI). Other manipulations include feminization of genetic males, male killing, and induction of parthenogenesis (Stouthamer et al., 1999; Bandi et al. 2001). The capacity of these bacteria to interfere with the host reproduction offers intriguing clues for the development of strategies for biocontrol of insect population and for interfering with insect-vector competence (Zchori-Fein et al., 2001). CI is particularly promising, because it has been proposed to efficiently drive a desired genetic trait in an arthropod population or as a method to suppress natural populations of insect pests in a way analogous to the sterile insect technique (Zabalou et al., 2004).

Very few investigations have been performed to date on the microbiology of the insect vectors of phytoplasmas in grapevine, and several considerations can only be done from studies performed in phylogenetically related insect models. Among the Hemiptera related to *S. titanus* and *H. obsoletus*, an insect that received strong attention for microbial symbionts is the glassy-winged sharpshooter *Homalodisca vitripennis* (formerly *H. coagulata*), the vector of *Xylella fastidiosa*, the causative agent of Pierce's disease of grapevine. *H. vitripennis*, a Cicadellidae of the same family of *S. titanus*, has been shown to host two major symbionts, the γ -Proteobacterium *Candidatus* *Baumannia cicadellinicola* (Moran et al., 2003) and the Bacteroidetes *Candidatus* *Sulcia muelleri* (Moran et al., 2005). These two symbionts were defined as two "coprimary" symbionts due to their long-term coinheritance during the diversification of the host (Takiya et al., 2006). Based on cocladogenesis and genome size evidences and on functions deduced from the genomes, Moran (2007) proposed that *Sulcia* became an obligate associate of an insect host that began to feed by sucking on primitive vascular plants when they appeared on earth, i.e., in the late Permian. Only much later, in the tertiary, following an adaptation of the host to feed on a xylem sap diet, the second obligate symbiont *Baumannia* appeared. This evolutionary reconstruction and the definition of coprimary symbionts are supported by the actual function of the two symbionts of *H. vitripennis*. Genome sequencing showed that these two symbionts play complementary roles for the host nutrition (Wu et al., 2006; McCutcheon and Moran, 2007). The very small, 245 kb genome of *Sulcia* retains pathways for the synthesis of most essential amino acids that are lacking in the xylem sap. *Baumannia*, with its 686 kb genome, retains the amino acid

synthesis pathways, lacking in the *Sulcia* genome, e.g., the histidine pathway, and those for biosynthesis of vitamins (Wu et al., 2006; McCutcheon and Moran, 2007).

Further studies have shown that besides the two primary symbionts, *H. vitripennis* hosts as a secondary symbiont *Wolbachia*, which was found to be the most frequently detected bacterium in the hemolymph of the glassy-winged sharpshooter (Takiya et al., 2006; Curley et al., 2007). Other studies have been performed on *H. vitripennis* from a more applied perspective for the control of Pierce's disease transmission. A culturable bacterium of the genus *Alcaligenes* that has been found associated with the insect vector of Pierce's disease and the grapevine plant has been proposed as a potential biocontrol agent for blocking the transmission of the disease through a paratransgenic approach (Bextine et al., 2004; Miller et al., 2006).

As far as the case of FD and BN, very little is known about the microbiota associated with the insect vectors in general and about the sexual endosymbionts in particular. The only sexual endosymbiont described to date associated with the vectors of phytoplasmas in grapevine is a *Cardinium* sp. that has been described in *S. titanus* (Marzorati et al., 2006).

Cardinium symbionts in *S. titanus* and *H. obsoletus*

Using a classical community fingerprinting approach that consisted of the application of LH-PCR with primers targeting the 16S rRNA gene of bacteria, PCR-DGGE and sequencing, Marzorati et al. (2006) identified a major symbiont of *S. titanus* that was affiliated to the genus *Cardinium*. By using a specific PCR, *Cardinium* was identified in almost all of 103 field-collected individuals of *S. titanus*, with a minimal field infection rate of 94.2%.

Cardinium associated with *S. titanus* had the closest 16S rRNA gene sequence identity and phylogenetic relationship with a symbiont of the tick *Ixodes scapularis* (98% identity). It grouped in a phylogenetic branch with endosymbionts of several species of the genus *Brevipalpus*, including the feminizing symbiont of *B. phoenicis* (Weeks et al., 2001, 2003), and of other acarine genera such as *Metaseiulus*, *Oppiella*, and *Petrobia* (Jeyaprakash and Hoy, 2004; Weeks et al., 2003). A bit more distant, in a separate branch, grouped *Candidatus Cardinium hertigii* endosymbionts of *Encarsia pergandiella* (Zchori-Fein et al., 2001, 2004), and endosymbionts of *Aspidiotus paranerii* (Weeks et al., 2003) and *Plagiomerus diaspidis* (Zchori-Fein and Perlman, 2004). Recent experiments performed in our laboratories with molecular ecology and microscopy techniques indicated that *Cardinium* is also hosted by *H. obsoletus* in the female reproductive system. Endosymbionts phylogenetically related to *Cardinium* have been observed in insects and Acarinae (both mites and ticks) (Kurtii et al., 1996; Zchori-Fein and Perlman, 2004; Enigl and Schausberger, 2007) and recently, intracellular structures with the same micromorphology of *Cardinium* cells have been found in the femoral organs of spiders (Pekár and Šobotník, 2007). Endosymbionts related to *Cardinium* have also been identified in the plant-parasitic nematode *Heterodera glycines* (Noel and Atibalentja, 2006). By using a TEM approach endosymbiotic cells with the same morphological signatures of *Cardinium* were detected in several tissues of the nematode. However, a phylogenetic classification indicated that the symbiont of *H. glycines* is sufficiently distant from *Cardinium* to be attributed to a new genus named *Paenicardinium* (Noel and Atibalentja, 2006). All these recent observations further suggest that this Bacteroidetes group might be even more widespread and diverse than thus far supposed.

The biological significance of the association between *Cardinium* and the different organisms has been addressed only in some cases. *Cardinium* has been shown to be associated with a variety of effects on the reproductive behavior (Kenyon and Hunter, 2007) and of reproductive alterations, including parthenogenesis, feminization of genetic males, and CI (Zchori-Fein et al., 2001, 2004). No data are currently available that indicate any bias in

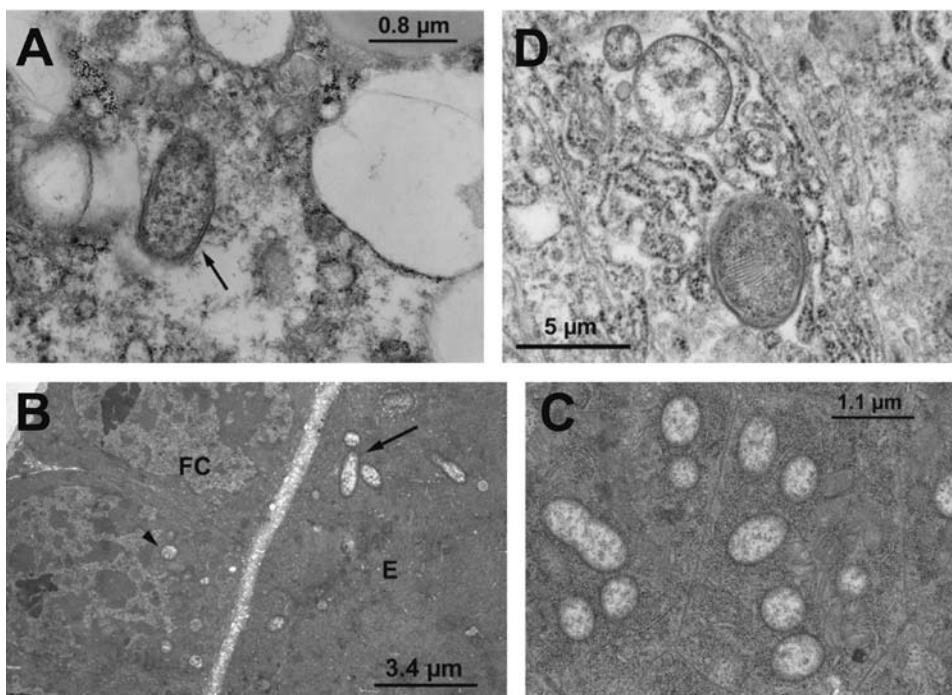


Figure 16.3 Localization of *Cardinium* sp. in the organs and tissue of *S. titanus*. (A) A *Cardinium* cell in the fat body. (B) Micrograph showing *Cardinium* in the female reproductive system. *Cardinium* cells are indicated by an arrowhead in the follicular cells (FC) and by an arrow in the egg (E). (C) Magnification of *Cardinium* cells in the egg cytoplasm. (D) A *Cardinium* cell in the salivary gland.

the sex ratio of *S. titanus* or *H. obsoletus*, and because the prevalence of *Cardinium* has been found similar in males and females, no obvious indication of interference with the sex ratio can be predicted. Such a high prevalence in both sexes of *S. titanus* could be the result of a selective sweep caused by CI (Stouthamer et al., 1999), or of a mutualistic interaction with the host.

Cardinium has been shown to be capable of colonizing several organs/tissues of *S. titanus* (Marzorati et al., 2006; Bigliardi et al., 2006; Sacchi et al., 2008; Figure 16.3). Examination by TEM of adult females indicated the presence of numerous *Cardinium* cells in the fat body (Figure 16.3A), suggesting that this symbiont may have a metabolic role for the host. *Cardinium* was also found in both the oocytes and the follicle cells of the ovary (Figure 16.3B and 16.3C), indicating that this bacterium is vertically transmitted to the offspring. Another very interesting localization of *Cardinium* within the body of *S. titanus* is in the salivary glands (Figure 16.3D). This localization, besides overlapping with that of phytoplasmas, opens the question of whether this bacterium might be transmitted to the plant during feeding and from the plant to other insect individuals. Horizontal transmission patterns for secondary sexual symbionts have been proposed several times based on the lack of evidence for cocladogenesis between (secondary) symbionts and their hosts, but to our knowledge there are very few reports documenting horizontal transmission of obligate symbionts (Huigens et al., 2001; Nussbaumer et al., 2006). For example, it has been reported that parthenogenesis-determining *Wolbachia* is horizontally transmitted

from infected to uninfected larvae of the egg parasitoid wasp *Trichogramma kaykai* while feeding on the butterfly host *Apodemia mormo deserti* (Huigens et al., 2001). On the opposite, Matalon et al., (2007) failed to find a horizontal transmission of *Cardinium* between the cactus scale *Diaspis echinocacti* and its parasitoids *Plagiomerus diaspidis* and *Aphytis* sp. and the hyperparasitoid *Marietta leopardina*. By using molecular ecology approaches, including fluorescence *in situ* hybridization, the authors were able to find *Cardinium* only in the parasitoid *P. diaspis* (Matalon et al., 2007).

A particular insect cell morphotype with the cytoplasm filled with *Cardinium* was found to be present in the apical region of the ovary (Sacchi et al., 2008). These cells resemble bacteriocytes, i.e., cells harboring symbiotic bacteria described in a variety of insects, including cockroaches and aphids (e.g., Sacchi and Grigolo, 1989; Nardon and Nardon, 1998). It has been proposed that the bacteriocyte-like cells play an active role in the transmission of the symbionts to the progeny (Sacchi et al., 2008), similarly to the bacteriocytes of cockroaches and the termite *Mastotermes darwiniensis* (Sacchi and Grigolo, 1989). In these insects another Bacteroidetes symbiont of the genus *Blattabacterium* lives within bacteriocytes that infiltrate the ovarioles, ensuring bacterial transmission to the oocytes. In *S. titanus* such a transmission pattern was supported also by the detection of (symbiotic) *Cardinium* cells in the initial phases of embryo development and during the third nymphal stages when bacterial cells were found in the cytoplasm of the oogonia (Sacchi et al., 2008).

When examined by TEM, *Cardinium* cell presents several peculiar morphological structures (Bigliardi et al., 2006; Sacchi et al., 2008; Figure 16.4), including a brush-like structure that resembles the parallel roads of ancient Roman towns (Zchori-Fein et al., 2004), i.e., the *cardi* (from which the genus name derives). *Cardinium* cell shows a two-layered envelope (an outer cell wall and an inner plasma membrane) and presents the already mentioned brush-like array of microtubule-like structures, which have been considered a morphological signature of the genus. The microtubule-like complex consists of a system of parallel microtubule elements, a fibrous electron dense plaque, and a set of electron dense structures adhering to the outer leaflet of the bacterial plasma membrane (Figure 16.4). The metabolic and physiological significance of this complex tubular structure is unknown; it might perhaps represent a membrane system where enzymatic activities occur.

A yeast-like symbiont in the body of S. titanus

Several Hemiptera, including aphids and planthoppers, have been shown to host, besides prokaryotes, intracellular eukaryotic microorganisms (Buchner, 1965; Noda, 1974; Chen et al., 1981; Ishikawa, 2003). One of the most studied models for the association with yeast-like symbionts (YLS) is the Asian rice brown planthopper *Nilaparvata lugens* (see for example Sasaki et al., 1996). It has been shown that in this planthopper the YLS was affiliated to Pyrenomycetes (now Sordariomycetes; Noda et al., 1995). In *N. lugens* and the other insect species thus far investigated, essential roles for the normal host development have been proposed, including recycling of nitrogen contained in the uric acid waste produced by the host by way of uricase enzymes (Chen et al., 1981, Sasaki et al., 1996; Hongoh and Ishikawa, 1997; Wilkinson and Ishikawa, 2001; Cheng and Hou, 2005). In the tobacco beetle *Lasioderma serricorne* a fungal gut endosymbiont detoxifies plant material ingested by the beetle (Dowd, 1989).

Very recently Sacchi et al. (2008) used molecular methods for the analysis of the fungal community associated with *S. titanus*. By using LH-PCR with primers targeting the fungal 18S rRNA gene they discovered that several fungal species were associated with the leafhopper. Among others (e.g., *Cladosporium cladosporioides*) that were supposed to be occasional commensal symbionts of *S. titanus*, sequences with 93% identity with *Bio-*

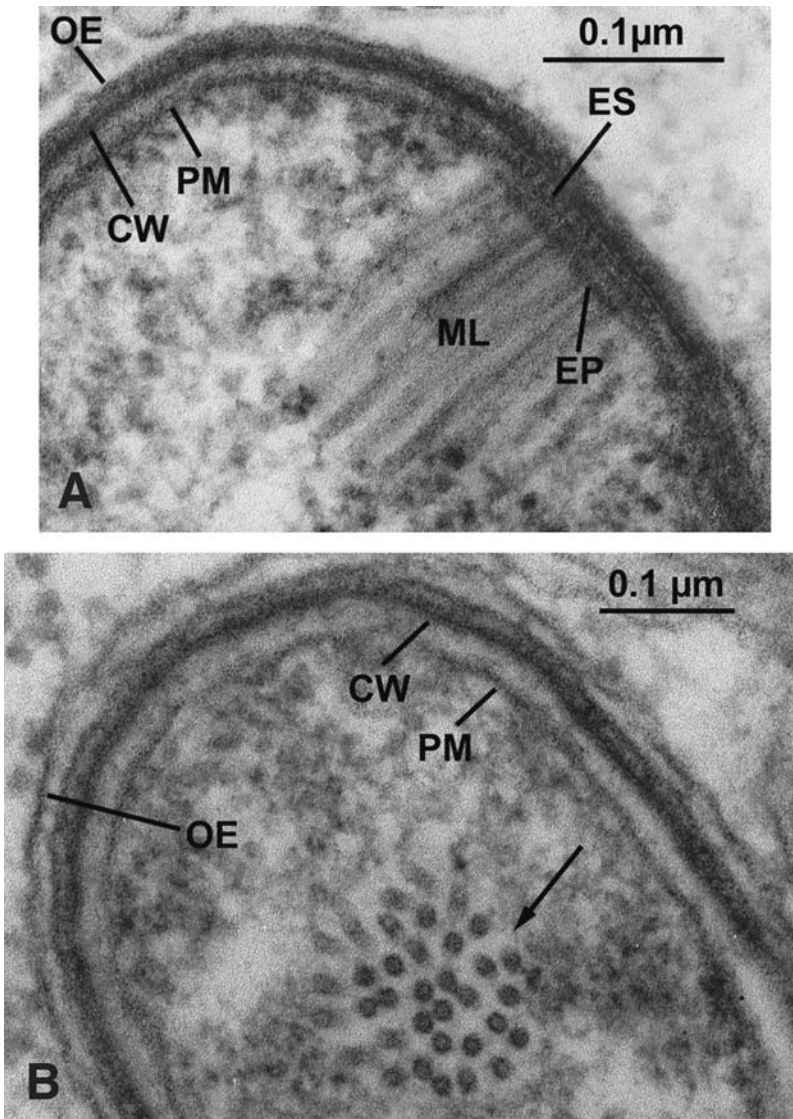


Figure 16.4 A particular ultrastructural morphology characterizes the cells of *Cardinium* sp., including the symbionts of *S. titanus*. (A) The brush-like structure as seen by a longitudinal view shows numerous microtubules (ML) inserted in an electron-dense plaque (EP) laying over a regularly distributed electron-dense structure (ES). An outer envelope (OE) that covers the cell wall (CW) over the plasma membrane (PM) is clearly visible. (B) A transversal view of the brush-like structure clearly shows that it is composed of microtubules (arrow). The outer envelope covering the cell wall and the plasma membrane could be the residue of an invagination process within the host cell membrane.

nectria pityrodes, a fungus belonging to the class of Sordariomycetes, were identified. PCR amplicons related to this fungus were observed in all of 32 *S. titanus* wild and greenhouse-maintained individuals, including males and females, indicating that this yeast is highly prevalent in the leafhopper. The presence of the symbiont was confirmed by *in situ* hybridization analyses that allowed the identification of the symbiont in the fat bodies of *S. titanus* (Sacchi et al., 2008).

S. titanus YLS appear to belong to the same phylogenetic lineage of the Ascomycotina that encompasses Sordariomycetes, even though a relatively low nucleotide identity (93%) with the closest relative in the databases has been found. Despite the fact that a longer sequence should be used to more carefully infer a precise phylogeny, the *S. titanus* YLS seems only distantly related with the already identified fungal symbionts of insects. It has been proposed that Sordariomycetes symbionts of Hemiptera stem from within the Cordyceps clade that contains obligate insect pathogens with filamentous growth (Suh et al. 2001). Such a consideration highlights the subtle evolutive borderline between parasite/pathogens and symbionts.

The micromorphology of *S. titanus* YLS as examined by TEM shows rod-shaped cells of $3 \times 15 \mu\text{m}$ in size with a two-layered cell wall composed of a first 25 nm-thick electron-dense layer and a second one 100 nm-thick and electron-clear (Sacchi et al., 2008). The YLS appeared to divide by budding, as in several cases cell protuberances typical of yeast during division process were found. High concentrations of YLS cells were found both in nymphs and adults, within certain specialized cells of the fat bodies (Figure 16.5A and 16.5B) that look like mycetocytes (Cheng and Hou, 2005). The very high number of YLSs observed in the fat body of *S. titanus* suggests that this microorganism plays a metabolic role that would possibly be linked to nitrogen recycling as already observed in other planthoppers like *N. lugens*. Indeed, we performed some experiments of rearing adult individuals of *S. titanus* in the laboratory on a diet based on sucrose solution without any nitrogen source. Many individuals were able to live in those conditions for periods of almost two months, which is equivalent to the typical adult life span in the field. Based on this evidence, it would be worthwhile to investigate further the possible role of the YLS in the nitrogen metabolism. Unfortunately, this kind of investigation is complicated by the fact that *S. titanus* is strictly monovoltine and its eggs must spend a long (but yet undefined) winter period at low temperature for hatching in spring.

In planthoppers and leafhoppers, models other than *S. titanus* YLS are vertically transmitted to the progeny following a transovarial route (Chen et al., 1981; Ishikawa, 2003). This is also the case of the vector of flavescence dorée (Sacchi et al., 2008). YLS cells could be observed by TEM in the process of infecting the ovary by passing from the hemolymph to the cells of the follicular epithelium and hence to the oocyte through an endocytotic process (Figure 16.5C and 16.5D). This pattern of localization in the ovary and the finding of YLS cells in the initial phase of the embryo development indicate the capability to be vertically transmitted (Sacchi et al., 2008). With respect to *N. lugens* eggs that host “symbiote ball” with a dense population of yeasts (Cheng and Hou, 2005), *S. titanus* ovary and young embryos contain a lower number of YLS cells. This envisages that the vertical transmission of the YLS in *S. titanus* has a lower rate than in *N. lugens*. The vertical transmission of the

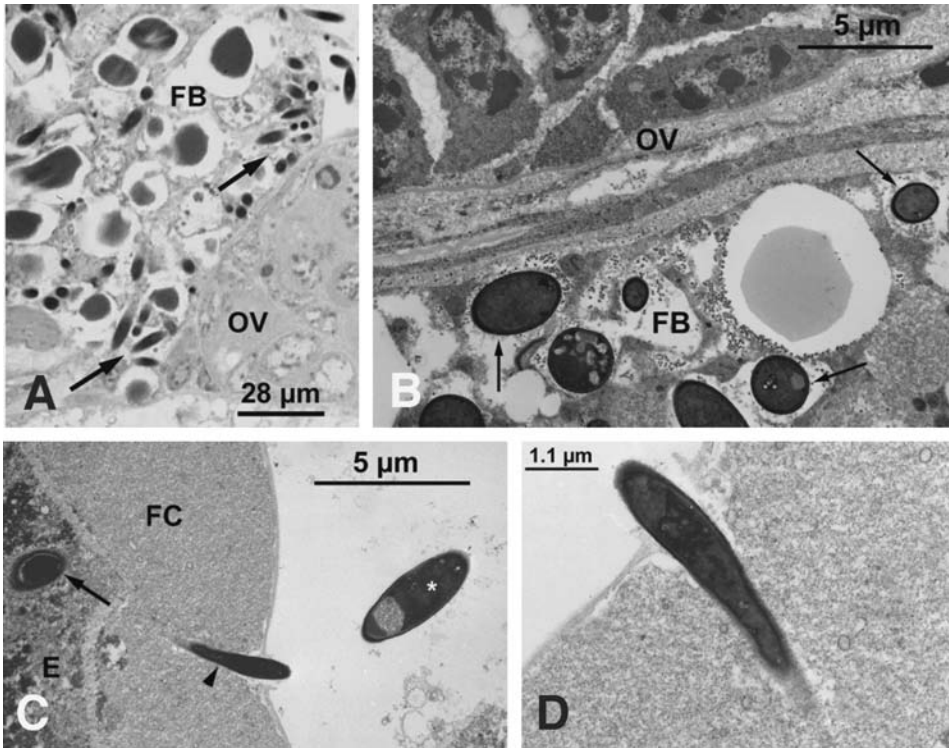


Figure 16.5 Localization of YLSs in the organs and tissues of *S. titanus*. (A) Several YLSs (arrows) are localized in the fat body (FB) close to the ovary (OV). (B) Micrograph of the region at the border of the fat body and the ovary (OV) showing YLS cells (arrows) in the fat body. (C) Three YLS cells in the process of passing from the hemolymph to the ovary. One cell is visible in the egg (E; arrow), a second one is in the hemolymph (asterisk), and a third (arrowhead) is in the process of entering a follicular cell (FC). (D) Magnification of a YLS cell entering a follicular cell of the ovary.

YLS seems to be in some way limited, possibly due to the large size of the YLS cells or to a potential competition for the transmission with the *Cardinium* bacterial symbiont.

Conclusions

In light of the recent developments in insect symbiont biology, there is a growing interest in the potential use of microbial agents for controlling insects, parasites, and the pathogens they transmit (Beard et al., 1998, 2002; Rio et al., 2004; Schnepf et al., 1998). Interference with pathogen vector capacity could be based on natural symbionts like the unharmed *Rickettsia peacockii* that appears to reduce the prevalence of the pathogen *Rickettsia rickettsii* when present in the tick *Dermacentor andersoni* (Baldrige et al., 2004), or could be achieved through the genetic manipulation of insect symbiotic microorganisms (Beard et al., 1998, 2002). Such strategy could have a future also in the case of phytoplasma-based disease only following careful and extensive investigations on the microbiota associated to the insect vectors. In the last few years, efforts on symbiont research were started for phytoplasma disease of grapevine, in particular for *S. titanus* (Marzorati et al 2006; Bigliardi et al., 2006;

Sacchi et al., 2008). Several candidate bacteria have been identified that could be used for biocontrol purposes. *Cardinium* appears very promising in the light of its localization in the same insect organs and tissues where the phytoplasmas pass through. In particular, the capacity to colonize salivary glands seems very interesting, also because an eventual transmission of the symbiont by the way of the plant could guarantee a multiple way of transmission of the biocontrol agent within natural vector populations. Other possibilities could be to exploit potential effects of *Cardinium* on the sexuality of *S. titanus*. However, the influence of *Cardinium* on *S. titanus*, if any, should be first clarified. Another interesting symbiont that raised interest for symbiotic biocontrol is *Asaia*, which has been found to be associated with mosquitoes (Favia et al. 2007) but also to hymenopteran parasitoids (Matalon et al., 2007). In mosquitoes it has been shown that this acetic acid bacterium can move within the mosquito body very efficiently and can easily pass through different individuals both vertically and horizontally with the diet and by paternal transmission (Favia et al., 2007). Finding similar acetic acid bacteria in *S. titanus* (Marzorati et al., 2006) suggests that a colonization pattern of the body similar to that of mosquitoes may occur in the leafhopper vector of FD, and opens the perspective for new research in the field of the biology of FD.

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Paratransgenesis in termites

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Introduction

Termites (order Isoptera) comprise over 2,700 species and are of global importance as decomposers of lignocellulose material (Kambhampati and Eggleton, 2000; König et al., 2006). Over 80% of the approximately 183 economically important termite species are subterranean termites, with the genus *Coptotermes* accounting for the largest number (28) of pest species (Su and Scheffrahn, 1998). In the United States, the need for control of the native Eastern subterranean termite (*Reticulitermes flavipes*) and the invasive Formosan subterranean termite (*Coptotermes formosanus*) supports a multimillion-dollar pest control industry (Su and Scheffrahn, 1998). The cost of control and repairs due to subterranean termite damage is estimated at over \$2 billion per year in the United States alone (Culliney and Grace, 2000).

Subterranean termites rely on beneficial symbioses with a diverse microbial flora in their guts to aid in digestion of lignocellulosic compounds in wood, which are their sole source of nutrition (Breznak, 2000; Brune, 2006). Even though cellulases are produced by subterranean termites in the salivary glands, foregut, and midgut (Nakashima et al., 2002), these endogenous cellulases alone are not sufficient to support the nutritional needs of a termite colony. Studies eradicating the intestinal flora of termites through antibiotics (Eutick et al., 1978) or oxygen (Veivers et al., 1982) indicate that important symbionts, which are vital for the survival of the termite host species, are among the microbial community. The main roles of the gut community, which consists of protozoa (Eucarya) and prokaryotes (Archaea, Eubacteria), are to supplement the termites' diet with nitrogen (nitrogen fixation and recycling), to aid in wood to digestion (cellulose degradation), and to provide

energy through metabolic pathways, for example, acetogenic reduction of CO₂ (Potrikus and Breznak, 1981; Breznak and Switzer, 1986; Waller, 2000; Bignell, 2000).

Because subterranean termites harbor a vast diversity of microorganisms in their guts and termite colonies are dependent on this symbiotic network to survive, the microbial community itself could provide much needed tools and targets for termite control. A novel pesticide-free approach to termite control could be derived from the use of genetically engineered gut symbionts that deliver and express toxins in the termite gut and then spread throughout a termite colony by social interactions (Husseneder et al., 2006; Husseneder and Collier, 2007).

Subterranean termite biology and control

A single colony of subterranean termites can contain millions of workers foraging throughout an area that can span a hundred meters or more (Su and Scheffrahn, 1998). Hidden nesting and feeding habits, large foraging areas (Husseneder et al., 2005b; Messenger et al., 2005), high reproduction rates in mature colonies (Su and Scheffrahn, 1987), and the potential of nymphs and workers to become reproductives when the previous generation of kings and queens is declining (Thorne et al., 1999; Vargo et al., 2003, 2006a, 2006b; Husseneder et al., 2005b, 2007) make subterranean termite control challenging. To eliminate an entire colony, it is necessary to kill not only the foraging population but also the reproductives in an efficient manner.

Traditional treatment for termites relies on soil or spot treatments with pesticides (Su and Scheffrahn, 1998). These chemical control methods pose certain risks of environmental contamination, nontarget effects, and the development of insecticide resistance. The need for reduction of chemical residuals in the environment has required that newly developed pesticides be biodegradable, and thus, their efficacies rapidly decrease (Su and Scheffrahn, 1998). Therefore, the public increasingly demands termite control technology that is not only environmentally friendly and target specific, but also sustainable long enough to effectively eliminate entire termite colonies.

One of the most successful reduced-risk approaches to termite control is the targeted bait approach using slow-acting toxicants, such as insect growth regulators (Su and Scheffrahn, 1998). Foraging workers consume the bait toxicant and transfer the active ingredient through the colony via social interactions, such as food exchange and grooming. Because the concentration of toxicant is diluted as it is passed among nest mates, a large number of foragers must consume sufficient amounts of bait toxicant to achieve successful colony elimination. Although the concentration of toxicant in the bait can be adjusted (Su and Scheffrahn, 1998), the uptake of sufficient amount of toxicant cannot be guaranteed because subterranean termites do not necessarily have stable foraging areas and may (in some cases) move frequently in and out of bait stations or abandon bait stations after disturbance (Aluko and Husseneder, 2007).

Theoretically, the efficacy of baiting systems could be further improved by employing live biological control agents, such as nematodes, viruses, fungi, and bacteria (Su and Scheffrahn, 1998; Culliney and Grace, 2000). Because the active ingredient in the bait would be live organisms, the agents in theory would be self-sustaining, self-replicating, and self-perpetuating within the colony. Although there is evidence that some entomopathogens kill insects in laboratory studies, most of them have largely failed to meet expectations in field trials (reviewed in Su and Scheffrahn, 1998; Culliney and Grace, 2000). In termites, the effectiveness of biocontrol agents is limited due to biological constraints and logistical problems of their application to social insects. For example, termites have few natural

pathogens and their pathogenicity is weak (Culliney and Grace, 2000). Usually, pathogens are not persistent in the environment of the termite colony because termites employ “hygienic” measures such as fumigating their nests with naphthalene to kill pathogens (Chen et al., 1998). Termites have the ability to avoid contact with pathogens, remove pathogens through grooming, and isolate infected individuals from the colony (Logan et al., 1990; Culliney and Grace, 2000). Termites also have an efficient immune system to eliminate infections with foreign pathogenic microbes (Rosengaus et al., 1999). It has been suggested that even the gut community protects termites against opportunistic invaders and pathogens (Veivers et al., 1982). Overcoming defensive mechanisms and delivering pathogens throughout an entire colony would require a large number of initially infected individuals and a high dose of inoculum, which is difficult to achieve. Mass production and reapplications of foreign pathogens required to achieve colony control would be expensive, time and labor intensive, and not necessarily justified by the poor performance, limitation of treatment to the immediate area of application, and the temporary treatment effects (Grace, 1997; Culliney and Grace, 2000). In summary, there is a need for developing more effective termite control techniques against subterranean termites.

Paratransgenesis

To avoid the negative selection of foreign pathogens by the termites’ hygienic behavior, immune system, and the protective network of the natural gut flora, microbes that are naturally associated with the target insect species (i.e., symbionts) could be employed as Trojan horses. According to Greek mythology, the Trojan horse was used to secretly shuttle enemy soldiers into the city of Troy, which was surrounded by impregnable walls. Once in the city, the soldiers destroyed Troy and its citizens. Modern science explores genetically engineered microorganisms, which serve as Trojan horses to deliver foreign genes into an insect host or an insect population (Beard et al., 2002). The technique of using microorganisms, such as viruses, fungi, or bacterial symbionts, as gene-drive and expression vehicles in a host organism is called paratransgenesis (Durvasula et al., 1997; Beard et al., 1998). An important improvement over foreign biocontrol agents would be that indigenous symbionts and closely related organisms should not trigger defensive or immune responses of the target host.

Several projects are currently aiming at the use of paratransgenic arthropods for prevention of microbe-related plant diseases (Bextine et al., 2003, 2005) and for blocking transmission of vector-borne diseases of animals and humans. For example, work is in progress to create mosquito populations unable to transmit the agents of malaria and dengue fever (Olson et al., 1996; Beard et al., 1998; Yoshida et al., 2001). A paratransgenic system using genetically engineered midgut symbionts for *in vivo* expression of trypanocidal peptides in tsetse fly to control sleeping sickness is under development (Rio et al., 2004; Hu and Aksoy, 2005). The most advanced model for the application of paratransgenesis is the genetic modification of the actinomycete gut symbiont (*Rhodococcus rhodnii*) of the triatomid *Rhodnius prolixus* (“kissing bug”) to express antimicrobial peptides against the protozoa *Trypanosoma cruzi*, which causes Chagas disease (Durvasula et al., 1997, 2003). All of the above studies aim to make a host insect refractory to transmitting a disease agent; however, they are not meant to achieve population control by decreasing the host’s survival. Paratransgenesis could be useful for pest control if its application leads to the destruction of the symbiotic gut flora that the host insect relies on for survival.

Husseneder and Grace (2005) suggested that subterranean termites would be ideal candidates for the development and application of a paratransgenic model system for

pest control. As wood-feeding insects, subterranean termites live on restricted diet with poor nutritional value. The termite workers, which are responsible for feeding the colony (Kumari et al., 2006), do not produce sufficient cellulases to digest wood and therefore are dependent on a diversity of microbial symbionts (protozoa, fungi, and bacteria) in their guts. Symbionts form a stable population in each termite worker and are spread among nest mates by routine trophallaxis and grooming, and through the mandatory refaunation of each worker by its nest mates after molting (McMahan, 1969). The symbionts in the gut of subterranean termites are mainly obligate, coevolved species that are highly specific to the host (Kudo et al., 1998; Hongoh et al., 2003, 2005; Shinzato et al., 2005; Fisher et al., 2007) but also include minor proportions of less specific microorganisms, such as the Enterobacteriaceae (Husseneder et al., 2005c; Fisher et al., 2007), that are common symbionts in other insects (Moran, 2001). The microbial diversity of the termite gut allows agents to be chosen for control purposes that range from generalist strains that can be easily engineered and applied to a broad variety of pest insects to strains that are highly specific for only certain target insect species and thus environmentally safe.

Proof of concept of the use of paratransgenesis in the Formosan subterranean termite

The Formosan subterranean termite, *Coptotermes formosanus*, is one of the most economically significant invasive termite species, and therefore, an important target for developing increasingly efficient control methods, preferably without the use of pesticides (Su and Scheffrahn, 1998). In a first experiment leading to proof of concept of paratransgenesis in subterranean termites, Husseneder et al. (2005a) genetically engineered an *Escherichia coli* laboratory strain with a plasmid that expressed genes for ampicillin resistance and green fluorescent protein (GFP) and fed the genetically modified bacteria to Formosan subterranean termite workers. Workers were screened for the presence of transformed *E. coli* by culturing their gut bacteria under selective conditions in liquid overnight cultures and on solid agar plates. The presence of the GFP reporter gene was confirmed visually by fluorescence, and by PCR amplification of the GFP gene. Although the experiment successfully established that termite workers ingested genetically modified bacteria rapidly through feeding on inoculated filter paper and transferred the bacteria among nest mates, the bacteria did not survive in the termite gut for longer than a week. Because field colonies of subterranean termites can be expansive (Su and Scheffrahn, 1998; Messenger et al., 2005; Husseneder et al., 2005b), a week is probably too short to guarantee spread of genetically modified bacteria and sufficient gene expression levels throughout an entire colony to achieve colony control.

To increase the stability of the population of genetically modified microorganisms in the termite gut, Husseneder and Grace (2005) subsequently genetically engineered bacteria that were isolated from the gut of Formosan subterranean termites. Naturally occurring bacteria derived from the termites' own indigenous gut flora should not trigger defensive or immune responses and should be well adapted to the living conditions and selective pressures in the termite gut (Dillon and Dillon, 2004). *Enterobacter cloacae*, a strain that was isolated in ample amounts from the gut of Formosan subterranean termite workers (Manesmann and Piechowski, 1989; Husseneder et al., 2005c), was genetically engineered as the prototype of a shuttle bacterium to express ampicillin resistance markers and GFP. Transgenic strains of the genus *Enterobacter* have previously been used for microbial control in other insect species (Watanabe et al., 2000; Kuzina et al., 2002).

Beard et al. (2002) and Durvasula et al. (2003) listed criteria necessary for a successful strategy using paratransgenesis. These criteria include the ability to (1) isolate and culture symbiotic bacteria from the host insect, (2) genetically engineer these bacteria without loss of fitness, (3) establish methods of reintroduction of genetically modified bacteria into the host, (4) express foreign gene products in the host, and finally, (5) spread the transformed bacteria throughout a target population, and (6) monitor potential environmental contamination.

The transgenic *E. cloacae* shuttle system meets the above criteria (Husseneder and Grace, 2005). For example, Enterobacteriaceae belong to the natural, stable flora of the termites, and are amenable to isolation, culture (Mannesmann and Piechowski, 1989; Husseneder et al., 2005c), and stable transformation with foreign genes without significant loss of viability. A method of delivery of genetically modified bacteria into laboratory colonies has been established: the bacteria were ingested by workers feeding on inoculated filter paper within a few hours, the GFP gene was expressed in the termite gut, and expression persisted in the termite gut for two months. Genetically modified bacteria were efficiently transferred throughout the termite colony, among workers and soldiers even when the donor (termites initially fed with transformed bacteria) to recipient (fed with water on filter paper only) ratio was low. First assessments of environmental spread of genetically engineered bacteria and possible gene transfer to soil bacteria were conducted in the laboratory by the use of GFP as a traceable marker gene. When termites that contained a population of GFP expressing *E. cloacae* were kept on soil, genetically modified bacteria were temporarily transferred into soil. However, these bacteria did not accumulate in soil but declined over the course of several weeks, which suggests limited survival capability of the genetically modified bacteria among the community of soil bacteria. Gene transfer was not observed because all fluorescent bacteria recovered from the soil had the same morphological and biochemical characteristics as the original *E. cloacae* strain (Husseneder and Grace, 2005). With this study, proof of concept has been established for the use of paratransgenesis to deliver and express foreign genes in termite colonies.

The paratransgenic system using GFP bacteria was designed solely as a proof of concept of successful transformation of indigenous symbionts and as a monitoring system for ingestion, long-term survival of genetically modified bacteria in the termite gut, and efficient spread among colony mates. To optimize the system for termite control, the following goals have to be achieved. Targets and target-specific toxins have to be identified. The toxins need to be highly effective against the target, e.g., kill the obligate symbionts that the termite relies on for survival or destroy the termite gut itself, and be slow-acting enough to allow spread throughout the termite colony. Genes for these toxins have to be synthesized and the host microorganism has to be genetically engineered to express these target-specific toxins in the termite gut. To increase environmental safety, microorganisms have to be identified that are termite specific and thus are unlikely to survive outside the gut. However, the bacteria have to be common in the gut of Formosan subterranean termites regardless of their geographic origin and have to be isolated and cultured in the laboratory.

Termite gut protozoa as targets

Cleveland's classical work (beginning in 1923) showed that subterranean termites were not able to digest cellulose without their hindgut symbionts and subsequently starved to death. The most important endosymbionts in the termite gut are xylophagous protozoa, which produce the majority of cellulases and ferment cellulose to acetate (Odelson

and Breznak, 1985; Bignell, 2000). The paunch of Formosan subterranean termite workers houses three species of flagellate protozoa: *Pseudotrichonympha grassii* (Hypermastigida), *Spirotrichonympha leidy* (Trichomonadida), and *Holomastigotoides hartmanni* (Oxymonadida) (Koidzumi, 1921; Yoshimura et al., 1995). These three protozoa species are found exclusively in *C. formosanus* (Yamin, 1979) and are obligatory for cellulose digestion and termite survival (Yoshimura, 1995; Yoshimura et al., 1995). When termite workers of several different colonies were fed on filter paper with the protozoicidal drug metronidazole (2 g/L), defaunation of their guts was observed within seven days. The defaunated termites died within six weeks, which is consistent with starvation (Raina et al., 2004). An environmentally safe way to eliminate termite colonies would be to develop a paratransgenic approach to specifically kill the protozoa in the gut of termite workers and thus starve the colony.

Antimicrobial peptides to kill gut symbionts

Lytic peptides, which are a ubiquitous part of the nonspecific immune system of eukaryotes, disrupt the membranes of bacteria and protozoa by forming channels that lead to cell death (Mutwiri et al., 2000; Boman, 1995, 2003). Because lytic peptides are largely inactive against the electrically neutral, cholesterol-containing cell membranes of higher eukaryotes (Javadpour et al., 1996; Kamysz et al., 2003; Boman, 2003), most lytic peptides have low or no toxicity to nontarget organisms such as beneficial insects, humans, and other mammals. Because of their mode of action (membrane disruption), the development of resistance mechanisms to lytic peptides is less likely than to chemical insecticides. The highly evolved natural lytic peptides, as well as synthetic derivatives, may provide an environmentally friendly alternative to chemical insecticides, because concentrations in the micromolar range are active against microorganisms (Wade et al., 1990) and do not leave toxic residues in the environment.

Because it has been shown that antimicrobial peptides can have activity against insect transmitted parasites (Shahabuddin et al., 1998), several paratransgenic approaches to control vector borne diseases involve the use of lytic peptides to kill the protozoan disease agent in the insect host. For example, Hu and Aksoy (2005) discussed a paratransgenic strategy of using *Sodalis glossinidius*, a midgut symbiont of the tsetse fly, to express lytic peptides that were characterized from the tsetse fly fat body to control trypanosome transmission by tsetse. The proposed host bacterium *S. glossinidius* shows high level of resistance against lytic peptides that are part of the innate immune system of the tsetse fly (Hu and Aksoy, 2005). Durvasula et al. (1997) genetically engineered *Rhodococcus rhodnii*, the endosymbiont of the triatomid *Rhodnius prolixus*, to express *Cecropin A*, a lytic peptide lethal to the protozoan *Trypanosoma cruzi*, which causes Chagas disease. Stable expression of the lytic peptide from the endosymbiont into the bug's hindgut resulted in dramatic reduction of the number of parasites. Although the use of lytic peptides in these projects aimed at eliminating disease-causing parasites from insects without toxicity to the host itself, the goal in termite control would be to destroy the symbiotic community in the worker gut and thus starve the termite colony.

To be able to test the efficacy of lytic peptides against protozoa in the gut of the Formosan subterranean termite *in vitro*, we isolated the three species of protozoa (Koidzumi, 1921) from the termite gut. Using sterile Trager U media at pH 7.0 (Trager 1934) sparged with 2.5% hydrogen, 5% carbon dioxide, and 92.5% nitrogen, protozoa cultures could be maintained in an anaerobic glove-box for more than 24 hours outside the termite gut. This technique enabled us to test the efficacy of lytic peptides against the termite gut protozoa in anaerobic conditions similar to the termites' hindgut environment. Following exposure to

concentrations of 50 μM of *L-Cecropin B*, *L-Melittin*, *L-Hecate*, and *D-Hecate* (Hancock et al., 1995; Mutwiri et al., 2000), protozoa were deformed and movement of the protozoa ceased. All three protozoa species died within 5–10 minutes following lytic peptide application in culture, whereas untreated protozoa cultures stayed alive for at least 24 hours and longer.

However, when the natural *L*-enantiomers of the lytic peptides were fed to termite workers, no defaunation was observed (Figure 17.1). The lytic peptides were possibly digested by general proteases while passing through the digestive tract. To test this assumption, synthetic *D*-enantiomers of *Hecate*, which are resistant to enzymatic degradation and inactivation (Wade et al., 1990), were fed to termite workers. When termites were fed a 500 μM *D-Hecate* solution, vesicles formed inside the protozoa, protozoan membranes ruptured (Figure 17.2a), and defaunation of the gut was complete within a few days (Figure 17.2b). Because *D*-peptides cannot be synthesized naturally by any living organisms, they are not useful in a paratransgenic system.

The majority of symbionts, including the protozoa, live in the anaerobic pouches of the hindgut and are largely protected from digestion because the hindgut is not likely to contain general proteases (Fujita et al., 2001). Therefore, we tested whether *L*-forms of lytic peptides could stay active in the hindgut. Using microinjection, approximately 0.5 μL of 10 mM Tris-HCl, pH 7.4 (control) or 500 μM lytic peptide solution was injected into the hindgut of termite workers. Death of all protozoa in the guts was observed within 72 hours of lytic peptide treatment (Figure 17.3). Termites were defaunated and died within a range

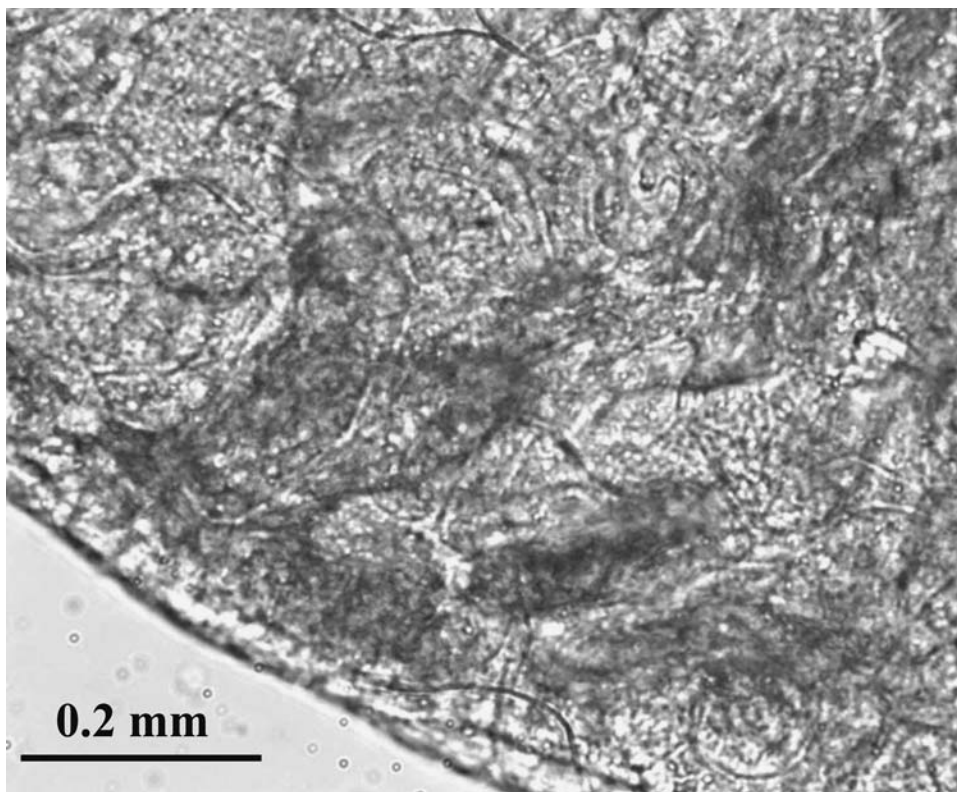


Figure 17.1 (Color figure follows p. 238.) Healthy termite gut containing a dense protozoa population.

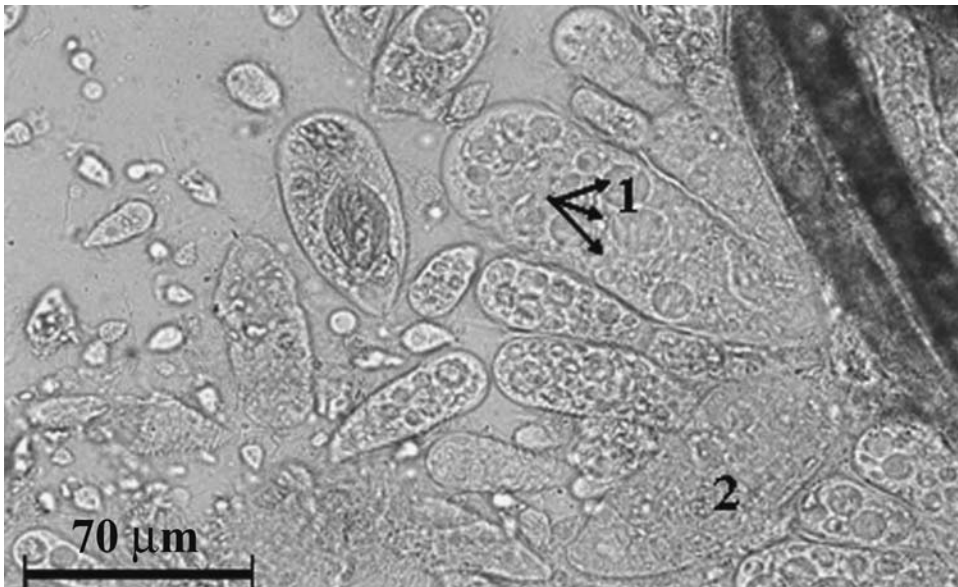


Figure 17.2a (Color figure follows p. 238.) Deterioration of protozoa in the hindgut after workers were fed *D-Hecate*. 1 = vesicles inside affected protozoan. 2 = dead protozoan.

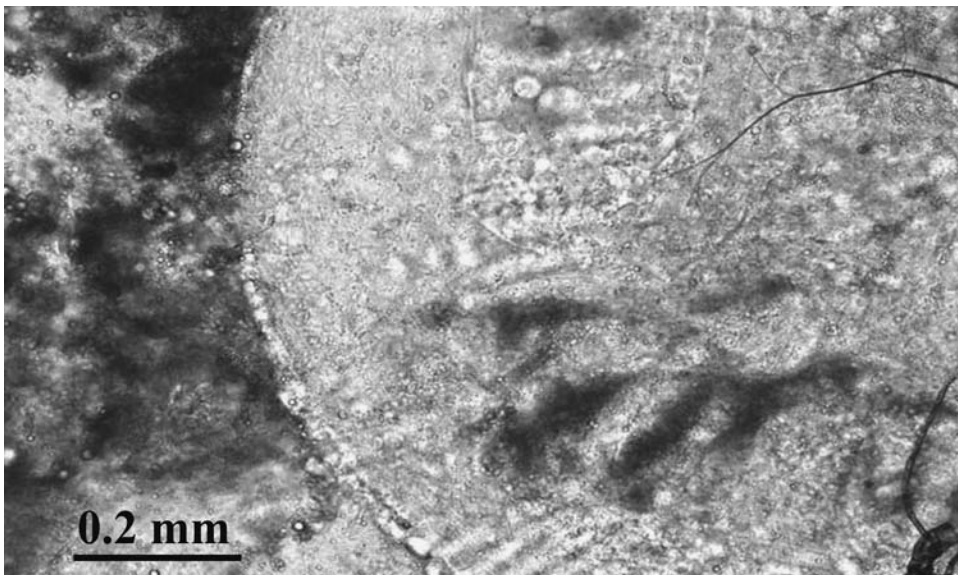


Figure 17.2b (Color figure follows p. 238.) Defaunated hindgut.

of a few days (*L-Melittin*) to 6 weeks (*L-* and *D-Hecate*, *Cecropin B*). The latter is consistent with death by starvation, as 6 weeks is approximately the life span of workers that have been defaunated with the protozoacidal drug metronidazole (Raina et al., 2004). The bee component venom *Melittin* is a very potent antimicrobial peptide. The accelerated death

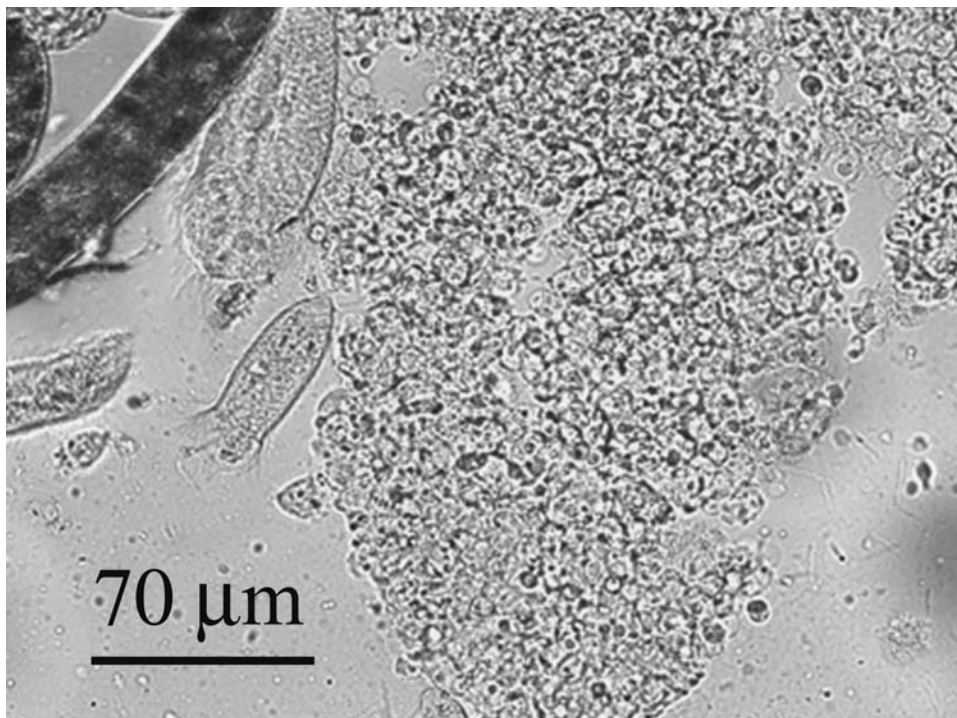


Figure 17.3 (Color figure follows p. 238.) Defaunation of worker hindgut after injection of lytic peptides.

after *Melittin* injection and observations of disintegration of the gut suggest that *Melittin* applied in concentrations of 500 μM and higher attacks not only the protozoa but also the termite gut itself. Therefore, the toxicity of *Melittin* against some eukaryotic cells (Boman and Hultmark, 1987) may prevent its application in a paratransgenic system. Although the application of *Melittin* should lead to a quick demise of individual termite workers, such a fast-acting toxin might not spread sufficiently among colony members to guarantee eradication of the entire termite colony. Slow-acting lytic peptides, such as *Hecate* and *Cecropin*, are therefore the toxins of choice to be delivered to and expressed in the hindgut by a paratransgenic microbial shuttle system.

Yeast as a prototype expression system for lytic peptides

Proof of concept for lytic peptide expression and secretion by a microbial shuttle system was achieved using a commercially available yeast-based expression system in *Kluyveromyces lactis* from New England BioLabs Inc. (Ipswich, MA). Successful ingestion of *K. lactis* by termites and survival in the hindgut was established by feeding yeast that was stained with nonlethal fluorescent yeast vacuole stain (Sigma-Aldrich, St. Louis, MO) to termite workers: presence of labeled yeast in the hindgut was visually confirmed under a fluorescence microscope.

The genes for *L-Hecate* and *L-Hecate* fused to green fluorescent protein (GFP) were codon-optimized for expression in *K. lactis* and chemically synthesized (Genscript Corp., Piscataway, NJ). The genes were cloned into the yeast's chromosome using an integration

vector (pKLACI). Untransformed yeast and a strain of *K. lactis* containing the integration vector but no lytic peptide encoding gene served as controls. Secretion of active lytic peptide by the transformed *K. lactis* into the growth media was verified in bioassays using *in vitro* cultures of the free-living laboratory ciliate *Tetrahymena pyriformis*. Live ciliates were counted after 24, 48, and 72 hours using a hemocytometer. Supernatants of growth media of two strains of *K. lactis*, one engineered to secrete *Hecate* and the other *Hecate-GFP*, produced high enough levels of the peptide to result in mortality of *T. pyriformis* that was significantly higher than that of the controls at 72 hours (Figure 17.4).

Subsequently, control strains and *Kluyveromyces lactis* strains that showed toxicity toward *T. pyriformis* were grown for three days at 30°C. Termites were fed with medium containing live yeast strains expressing *Hecate* or, *Hecate-GFP* or with control strains for one day and then kept on damp filter paper. Workers that were fed with the yeast strains expressing lytic peptide were defaunated at four weeks, whereas the control continued to contain active protozoa of all three species in high population density in their guts.

Aliquots of the yeast strains containing lytic peptide genes were maintained in culture and tested for loss of activity over time in bioassays with *Tetrahymena*. Approximately eight weeks after the initial confirmation of their protozoocidal activity, the yeast strains did not produce sufficient active lytic peptide to cause mortality in *T. pyriformis*. This suggests that expression of lytic peptide inflicts negative selection pressure on the yeast itself, resulting in genomic recombination and the loss of lytic peptide expression, which would increase

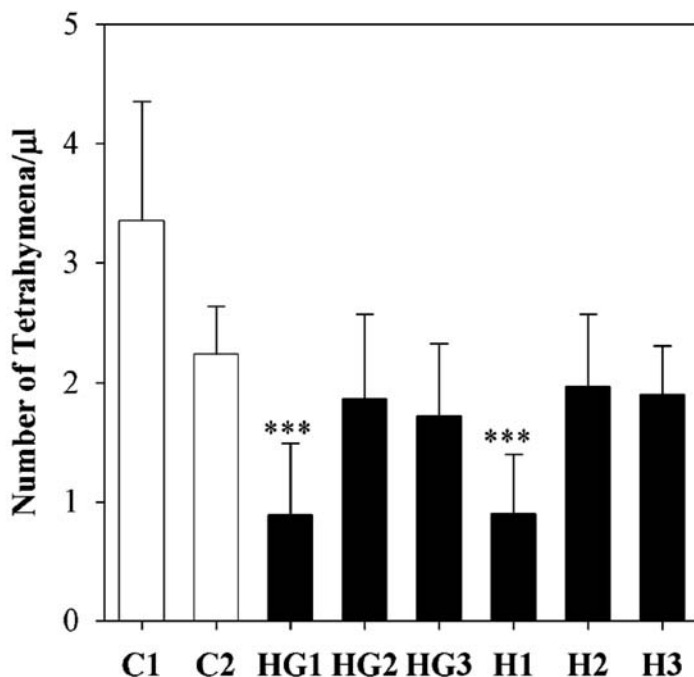


Figure 17.4 Mortality of protozoa exposed to lytic peptide expressing yeast. C1 = control with no yeast. C2 = control with yeast containing the vector, but no lytic peptide gene. HG1-3 = Three different yeast strains containing *Hecate-GFP* fusion genes. H1-3 = three different yeast strains containing *Hecate* genes. *** Significant reduction in the cell count of *T. pyriformis* compared to both controls ($p < 0.0001$, SAS Proc Mixed ANOVA, Tukey's mean separation).

environmental safety. To increase environmental safety further and protect the expression system from negative selection in the future, work is in progress to construct fusion genes that cause the yeast to secrete inactive protoxins, i.e., lytic peptides inactivated by C- and N-terminal peptide caps. The lytic peptides would be activated in the termite gut by cleavage of the caps with gut-specific proteases.

Additional target specificity could be achieved by the identification of unique receptors on the membranes of the termite protozoa, which would allow the development of protozoa-specific ligands to which the lytic peptides could be fused. Initial investigations focused on the identification of insulin receptors. Insulin-like receptors have been identified in *T. pyriformis* (Leick et al., 2001) and termite protozoa have been observed internalizing fluorescent insulin (fluorescein isothiocyanate conjugate of human insulin, Invitrogen Inc.) under the GFP microscope. Using the phospho-Insulin Receptor β Subunit (pTyr¹¹⁵⁸) ELISA (Sigma), we were able to identify insulin receptors on *T. pyriformis*. However, we did not detect insulin receptors on the termite protozoa using this method. Further studies will be required to identify surface receptors of the flagellates in the termite gut.

Bacterial diversity in the gut of the Formosan subterranean termite

A prototype gene-shuttle was assembled in yeast and used in termite feeding experiments; death of the protozoa in the termite gut provided proof of concept for the use of paratransgenesis in termite control. The ultimate goal for environmentally safe field applications, however, is to use novel bacteria that are exclusively found in the termite gut as shuttles and expression systems. Termite-specific bacteria species would not be able to survive for a prolonged time outside the termite gut and thus reduce the potential environmental impact, including the persistence of the transgenic bacteria in soil and possible gene transfer between bacterial strains.

Bacterial diversity has been studied in several subterranean termite species, for example, *Reticulitermes speratus* (Ohkuma and Kudo, 1996; Hongoh et al., 2003), *R. flavipes* (Fisher et al., 2007), and *C. formosanus* (Shinzato et al., 2005; Husseneder et al., 2005c) using culture-independent methods such as sequencing or restriction fragment length analysis of 16S rRNA genes. Shinzato et al. (2005) found 49 bacteria species in the guts of Formosan subterranean termites in Japan, including 39 novel species. Most of the remaining species belonged to the phyla *Bacteroidetes*, *Firmicutes*, and *Spirochaetes*. We are currently compiling an inventory of bacterial species found in Formosan subterranean termite colonies from its introduced range, i.e., Louisiana, Hawaii (Husseneder et al., 2005c) and Japan (Shinzato et al., 2005) and from its native range, i.e., China (Ho and Husseneder, 2007) to identify species that are obligate and common across geographical regions. To date, over 220 bacteria species from ten bacterial phyla were identified; over 80% were novel species, yet the majority clustered closely with bacteria lineages found only in subterranean termites and not in the environment (Hongoh et al., 2003; Shinzato et al., 2005). The diversity of bacteria in the termite gut provides a wide range of “raw material” for genetic transformation and use as a Trojan horse. The fact that most of these bacteria are specific to termites and are not likely to survive outside of the termite gut would increase environmental safety.

The ideal host bacterium for a paratransgenic approach would be a novel species that is culturable, but unlikely to survive in the environment for a prolonged period of time, and common in all colonies of the Formosan termite regardless of geographical region, but specific to this termite species. A novel *Bacteroides* species, which was dominant in the bacterial inventory of Formosan termites introduced to Japan (Shinzato et al., 2005), also was dominant in colonies in Louisiana (Husseneder et al., 2005c) and present in lower propor-

tions in Chinese colonies (Ho and Husseneder, 2007). However, this species has not been cultured yet. The most prominent culturable species that was found in Formosan subterranean termite colonies from Japan, Louisiana, and China was *Pilibacter termitis*, a novel species, which had been previously isolated from colonies in Hawaii (Husseneder et al., 2005c) and was described by Higashiguchi et al. (2006). *Pilibacter termitis* is a Gram-positive, lactic acid bacterium. Gram-positive bacteria are considered less susceptible to the membrane disruptive activity of lytic peptides than Gram-negative bacteria (Javadpour et al., 1996). Thus, *Pilibacter termitis* is a promising candidate for genetic transformation to become a bacteria shuttle. A limitation to the use of termite-specific novel bacteria species, such as *P. termitis*, is that protocols for genetic manipulation of this species do not exist in the literature. Transformation protocols are currently being developed based on methods used on phylogenetically similar bacteria. Even if the genetic transformation of *P. termitis* should not be successful, dozens of less frequent bacterial strains that were previously cultured from the diverse gut flora of the Formosan subterranean termite could be genetically engineered. These cultured bacteria range from novel species to common Enterobacteriaceae (Mannesmann and Piechowski, 1989; Osbrink et al., 2001; Husseneder et al., 2005c).

Alternatively, yeast remains a promising candidate host for the paratransgenesis system, especially with safety precautions, such as the addition of a ligand targeting the lytic peptide to the surface of the protozoa or the gut wall or with the activation of the secreted lytic peptide by termite-specific gut enzymes. The use of yeast as the Trojan horse has the advantage that it could be incorporated in existing bait systems in freeze-dried form, resulting in a long shelf life and easy application to termite colonies.

Summary

Paratransgenesis takes biocontrol one step further by genetically fortifying microorganisms to become a Trojan horse and spread detrimental gene products through pest populations, such as subterranean termite colonies. Living microbial agents can establish a self-sustaining, self-replicating, and self-perpetuating population that is transferred throughout an entire colony. Paratransgenesis may become a technology leading to a paradigm shift in termite control as soon as products are successfully developed and environmental safety is guaranteed.

We have established proof of concept for ingestion and transfer of genetically engineered bacteria by subterranean termites, and the long-term stability of gene expression in the termite gut using reporter genes. We have developed a prototype of a Trojan horse using yeast that expresses lytic peptides, which kill the protozoa in the termite gut and cause the termite colony to starve. Steps to increase environmental safety of the paratransgenic system are currently being developed. These developments include the use of termite-specific bacteria, which are unlikely to survive outside the termite gut, activation of the toxin by proteases specific to the termite gut, and ligands to target the toxin to receptors on the surface of the protozoa.

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chapter eighteen

Insect facultative symbionts: biology, culture, and genetic modification

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Introduction

Maternally transmitted, symbiotic bacteria have been identified in a wide range of insect taxa (Buchner, 1965). Although a few of these symbionts are known to be parasitic (e.g., *Wolbachia* spp.), the majority appears to have a beneficial effect upon their hosts. These mutualistic associations can be further classified as obligate or facultative, based on the level of dependence exhibited by the host insect. In an obligate association, the symbiont is anticipated to provide benefits that are essential for host survival and reproduction. In a facultative association, the symbiont is anticipated to provide benefits that merely enhance host fitness, sometimes under specific circumstances. Often, but not exclusively, the obligate associations are based on nutrition; symbionts provide essential nutrients that are either absent or restricted in the host’s natural diet.

Symbioses that are obligate and nutritional in nature include the most ancient associations that have been described to date (estimated to be 200–300 MY old; Gil et al., 2002; Nakabachi et al., 2006; McCutcheon and Moran, 2007). In these examples host–symbiont phylogenies often display high levels of congruence, reflecting a long history of strict matrilineal symbiont transmission and host–symbiont cospeciation. Facultative associations are typically more recent in origin (typically <100 MY old) and the phylogenies of hosts and their symbionts often lack concordance as a result of (infrequent) horizontal symbiont transmission events (Dale and Moran, 2006). Furthermore, because natural selection favors the streamlining of symbiont genomes over time, the gene inventories of ancient obligate symbionts tend to be smaller than those of facultative symbionts (Dale et al., 2006). Organisms with larger genomes are expected to be more biochemically (metabolically) plastic and consequently capable of survival under a wider range of environmental conditions.

In this chapter, we focus on the biology, culture, and genetic manipulation of insect facultative symbionts that reside in a wide range of insects. Over the past decade, we have witnessed a surge in interest in these organisms, driven by some interesting discoveries that provide insight into their *raison d'être*. At the same time we have made great strides in the development and application of laboratory techniques that allow these organisms to be cultured and manipulated outside of their natural hosts. Ultimately, these technical innovations are expected to facilitate (1) the use of genetic techniques to investigate the mechanisms of symbiosis (Pontes and Dale, 2006), and (2) the use of symbionts as a platform to express transgenes in insects of medical and agricultural importance (Durvasula et al., 1997).

Biology of facultative symbionts

Identification

The development of PCR revolutionized the study of insect symbionts by providing the opportunity to establish the identities of uncultured symbionts. Typically, degenerate (universal) oligonucleotide primers are used to amplify a subsection of the small subunit 16S rRNA gene, which is both ubiquitous and conserved across members of the bacterial domain. Once amplified, the 16S rRNA fragment can be sequenced directly or cloned into a plasmid vector, prior to sequencing. The 16S rRNA sequences obtained can be used to establish the identity of bacteria using the Basic Local Alignment Sequence Tool (BLAST). This tool compares the query sequence to all other sequences in the GenBank database to identify the most closely related sequences (98% similarity in 16S rDNA sequence identity signifies identity at the species level). Second, 16S rRNA sequences can be used to infer the evolutionary relationships between organisms through phylogenetic analysis. In some cases, however, closely related taxa may not differ sufficiently in their 16S rRNA sequences to resolve their relationships based on phylogeny, because of the slow rate of evolution of the 16S rRNA sequence. To overcome this problem, alternative genes can be amplified by PCR. The genes typically used for this purpose are “informational genes” (i.e., genes whose protein products are involved in transcription, translation, and DNA replications). These sequences are also ubiquitous and well conserved across bacteria but they tend to evolve more rapidly than 16S rRNA because of their degenerate third codon positions. In phylogenetic analyses, this increased evolutionary rate provides greater resolution of closely related taxa, because the sequences incur a larger number of substitutions per unit time.

Table 18.1 Representative Facultative Insect Symbionts

Symbiont	Hosts	Functions	References
<i>Ca. Hamiltonella defensa</i>	Aphids, whiteflies, psyllids	Defense against parasitoids	Chen and Purcell, 1997; Oliver et al., 2003, 2005; Moran et al., 2005a, 2005b
<i>Ca. Regiella insecticola</i>	Aphids	Host plant specialization, dispersal and mating, defense	Tsuchida et al., 2004; Moran et al., 2005b; Scarborough et al., 2005; Leonardo and Mondor, 2006
<i>Ca. Serratia symbiotica</i>	Aphids	Thermal tolerance, defense against parasitoids	Montllor et al., 2002; Koga et al., 2003; Oliver et al., 2003, 2005; Moran et al., 2005b; Russell and Moran, 2006
<i>Sodalis glossiniidius</i>	Tsetse flies	B vitamin synthesis	Nogge, 1981; Dale and Maudlin, 1999; Geiger et al., 2005
<i>Arsenophonus</i> spp.	Wasps, louse flies, whiteflies, aphids, ticks, triatomines	Unknown	Ghera et al., 1991; Hypsa and Dale, 1997; Grindle et al., 2003; Thao and Baumann, 2004; Dale et al., 2006

Distribution and transmission

Using the PCR-based identification techniques described above, facultative symbionts have been identified in a wide range of insects that often also harbor ancient obligate nutritional symbionts (Table 18.1). In almost all of the examples studied to date, the ancient obligate symbionts are found to evolve in strict concordance with their insect hosts. This results from long-term matrilineal transmission—as a given insect group speciates, its symbiotic bacteria are carried into the descending lineages and generations. Over evolutionary time, this leads to increased specialization and dependence for both partners in the symbiotic association. At some point in time, the genomic and metabolic attributes of the symbiont become so highly specialized that it cannot “escape” and colonize a novel insect host.

Not surprisingly, when we compare the phylogenetic trees of facultative symbionts and their insect hosts, there is often discordance. Although we can find examples in which closely related facultative symbionts inhabit closely related insect hosts, further exploration often reveals that those symbionts have a more widespread distribution. For example, almost identical strains of *Sodalis glossiniidius* have been identified in five closely related tsetse species (Aksoy et al., 1997), but close relatives of these bacteria have also been identified in hippoboscids flies, grain weevils, and bird lice (Heddi et al., 1998; Nováková and Hypsa, 2007; Fukatsu et al., 2007). In other cases, closely related insect species are found to harbor a number of different facultative symbionts. For example, aphids are known to harbor several distinct facultative symbionts from the family *Enterobacteriaceae* (Moran et al., 2005b). Finally, there are some examples of distantly related hosts that are known to harbor extremely very closely related symbionts. For example, representatives of the candidate genus *Arsenophonus* (sharing c. 99% identity in 16S rRNA) have been identified in many different arthropods, including parasitoid wasps (Hymenoptera), triatomine bugs,

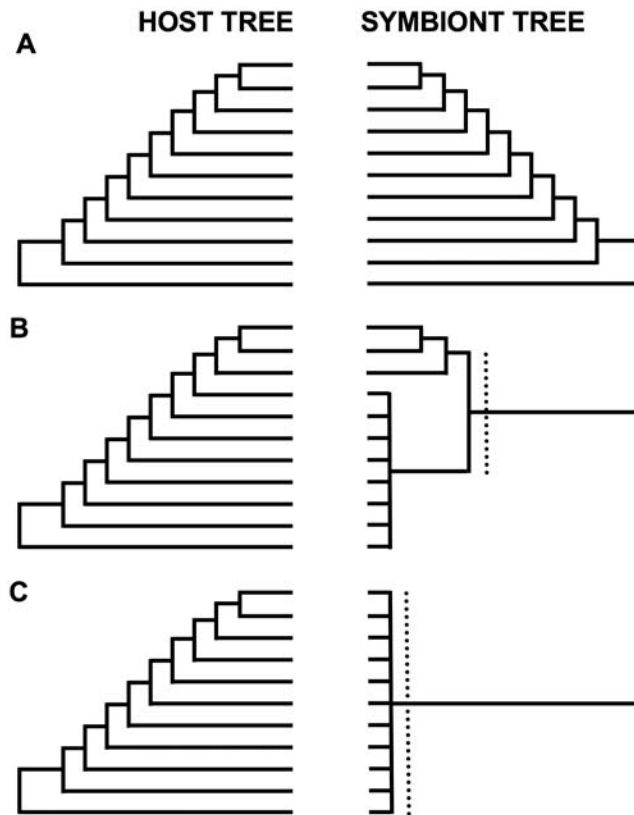


Figure 18.1 Three conceptual scenarios illustrating evolutionary relationships between insect hosts and their symbiotic bacteria. (A) Host and symbiont phylogenies show perfect congruence as a result of long-term cospeciation. This relationship is expected in ancient associations involving obligate, maternally transmitted, nutritional symbionts. (B) Partial congruence between host and symbiont phylogenies. The symbiotic association is more recent in origin and there is evidence of horizontal symbiont transfer between distantly related insect hosts (illustrated by dotted line). (C) No congruence observed between host and symbiont phylogenies. Symbionts have been horizontally transferred between all host species.

aphids, psyllids, whiteflies (Hemiptera), and ticks (Ixodida) (Gherna et al., 1991; Hypsa and Dale, 1997; Spaulding and von Dohlen, 2001; Grindle et al., 2003; Thao and Baumann, 2004; Dale et al., 2006).

The apparent contrasts observed in the distribution profiles of facultative symbionts can readily be explained in the context of symbiont transmission. Vertical (or maternal) transmission represents the predominant route by which facultative symbionts are transferred to the next generation. The exact mechanism of vertical transmission varies according to the reproductive strategy of the host insect (sexual or parthenogenetic, viviparous or oviparous), but the general procedure involves the transfer of symbiotic bacteria from maternal reproductive structures into eggs, embryos, or larvae. In the case of ancient, obligate symbionts, perfect vertical transmission over a long period of time gives rise to congruent host–symbiont phylogenies (Figure 18.1A). This occurs when an ancient symbiont infection event is followed by a period of host speciation—in the absence of horizontal transfer,

the symbionts follow the same pattern of descent as their hosts. Facultative symbioses are typically more recent in origin, but it is not unprecedented for their phylogenies to demonstrate some degree of congruence. Often this limited level of congruence is coupled with evidence of horizontal symbiont transfer into more distantly related host lineages, as depicted in Figure 18.1B. More frequently however, the phylogenetic trees of facultative symbionts display little or no congruence in comparison with host trees (Figure 18.1C). This is indicative of the recent acquisition of the symbiont by the insect host, and/or an increased frequency of horizontal transfer. Note that, over evolutionary time, only a very small number of horizontal transfer events are needed to abolish congruence between host and symbiont trees.

Several hypotheses have been proposed to explain the mechanics of horizontal symbiont transmission, but it seems likely that there is no single mechanism that accounts for this phenomenon in nature. One hypothesis states that facultative symbionts are horizontally transferred through host tissues during episodes of feeding (Darby et al., 2001). An alternative hypothesis states that insect parasitoids (e.g., hymenopteran wasps) play a role in horizontal transmission, based on the observation that closely related facultative symbionts are found in parasitoids and their corresponding insect hosts (Hypsa and Dale, 1997; Russell et al., 2003). Finally, in a recent study Moran and Dunbar (2006) provided experimental evidence of paternal symbiont transfer between aphids. This mechanism of horizontal transmission could occur in any insect species that undertake sexual reproduction, regardless of diet, niche, or parasitism. However, paternal transfer of symbionts is unlikely to account for the horizontal transmission of symbionts between distantly related insect species.

The horizontal transmission of facultative symbionts is dependent upon the ability of these bacteria to infect naïve hosts—a characteristic that has likely been lost in ancient obligate symbionts. Two lines of evidence indicate that facultative symbionts have the capability to colonize a (compatible) naïve host insect. First, facultative symbionts have been successfully transferred from infected hosts into uninfected hosts by microinjection of symbiont-infected hemolymph (Oliver et al., 2003; Russell and Moran, 2005, 2006). Second, cultured recombinant strains of *S. glossinidius* have been microinjected into tsetse flies and found to be maintained in subsequent host generations (Dale et al., 2001; Weiss et al., 2006). Interestingly, these studies have also identified a number of genes that symbionts use to facilitate host cell invasion (Dale et al., 2001, 2005; Moran et al., 2005a).

Localization

The ability to invade cells explains why facultative symbionts are not confined exclusively to specialized bacteriocytes. Facultative symbionts have been identified in insects in many different locations including secondary bacteriocytes, sheath cells, fat body, neural ganglion, salivary glands, reproductive tissues, and hemolymph (Hypsa and Dale, 1997; Moran et al., 2005b; Dale et al., 2006). Due to their broad distribution within host tissues, care needs to be exercised in the identification and localization of facultative symbionts. Although basic microscopic techniques can be used to stain and visualize bacterial cells in tissue sections, they fail to provide a means for identification.

Fluorescence *in situ* hybridization (FISH) is a powerful technique that facilitates identification and localization of symbionts in host tissues (Amann et al., 1991; Fukatsu et al., 1998). This technique relies upon the use of labeled oligonucleotide probes that provide specificity at the level of a bacterial species or strain. Fluorescently tagged 16S rRNA probes are hybridized to tissue sections *in situ*, and viewed under an epifluorescence microscope

for precise localization. FISH has been used successfully on pea aphid tissue sections to localize facultative symbionts in secondary mycetocytes, sheath cells, and hemolymph as well as in male reproductive tissues (Tsuchida et al., 2005; Moran and Dunbar, 2006). In addition, researchers using FISH discovered an unusual case of γ -proteobacterial symbionts residing within β -proteobacterial symbionts in mealybugs (von Dohlen et al., 2001). One problem inherent in using FISH in this manner is that some insect tissues tend to exhibit high levels of autofluorescence, and this leads to the masking of the fluorescent signals obtained from the hybridized probes (Fukatsu et al., 1998). In some cases these problems have been overcome using digoxigenin or biotin labeled probes instead of fluorescent probes, and this seems to mitigate the problems caused by tissue autofluorescence (Fukatsu et al., 1998, 2000).

Role of facultative symbionts

Whereas most obligate symbionts of insects play an essential role in supplementing the host diet, the roles of the facultative symbionts are more varied (Table 18.1). Some facultative symbionts do seem to have a nutritional function within their host insect. *Sodalis glossinidius* is thought to play a role in nutrition by supplementing the diet of its tsetse fly host with essential B vitamins that are lacking in the host's natural diet of vertebrate blood (Nogge, 1981). The specific elimination of *S. glossinidius* from the tsetse host leads to a decrease in host longevity, concomitant with the loss of nutritional supplementation (Dale and Welburn, 2001).

Other facultative symbionts appear to have roles unrelated to host nutrition. One of the best-studied systems is that of the pea aphid, *Acyrtosiphon pisum*, and its complement of facultative symbionts. *Acyrtosiphon pisum* strains always contain an obligate, bacteriocyte-associated endosymbiont, *Buchnera aphidicola*, which supplies the host with essential amino acids lacking in its phloem diet (Douglas, 1998). In addition, many pea aphids typically harbor one or more facultative symbiont(s), most notably *Candidatus Hamiltonella defensa* (alternately called PABS or T-type), *Candidatus Regiella insecticola* (PAUS or U-type), or *Candidatus Serratia symbiotica* (PASS or R-type; Chen et al., 1997; Darby et al., 2001; Moran et al., 2005b). The nature of the relationship between these facultative symbionts and the host insect has been a topic of much study in recent years. Recently, clonal lines of pea aphids lacking facultative symbionts (aposymbiotic) have been established for use as recipients in microinjection experiments. Small amounts of hemolymph from symbiont-infected strains are transferred to these aposymbiotic strains to establish artificially infected lines. The uninfected and artificially infected lines provide an excellent basis for the comparative evaluation of symbiont-host interactions. These studies have shown that the three aphid facultative symbionts described above play a variety of roles in the insect host under distinct ecological conditions. For example, aphids infected with *Ca. Hamiltonella defensa* or *Ca. Serratia symbiotica* show enhanced resistance to parasitism by *Aphidius ervi*, a parasitoid wasp that commonly preys on aphids (Oliver et al., 2003, 2005; Moran et al., 2005a). In addition, *Ca. Serratia symbiotica* benefits its aphid host by enhancing thermal tolerance and compensating for loss of the obligate symbiont, *B. aphidicola* (Montllor et al., 2002; Koga et al., 2003). *Candidatus Regiella insecticola* seems to enhance aphid fitness on particular plant species (Leonardo and Muir, 2003; Tsuchida et al., 2004) and also plays a role in protecting aphids from fungal infections (Scarborough et al., 2005).

In addition to determining symbiont functions, there is also interest in understanding interactions that occur in insects with superinfections (multiple species of facultative

symbionts in one host). For example, aphid facultative symbionts appear to have many beneficial effects, and one might postulate that aphids would maximize their fitness by acquiring as many different facultative symbionts as possible. However, in wild aphid populations superinfections are rarely observed (Oliver et al., 2006). This is likely because of trade-offs encountered in balancing the advantages of beneficial symbionts with the disadvantages of harboring large numbers of bacteria. This was demonstrated in a study by Oliver et al. (2006) in which aphids superinfected with *Ca. Hamiltonella defensa* and *Ca. Serratia symbiotica* showed increased resistance to parasitism at the expense of host fitness.

Facultative symbionts also have the potential to interact with other organisms harbored within the host insect. Tsetse flies, *Glossina* spp. are vectors of African trypanosomes, the causative agent of sleeping sickness in humans and nagana in their cattle. The presence of *S. glossinidius* is linked to both an increase in tsetse longevity, and an increase in the frequency of establishment of trypanosome infections within the tsetse host (Dale and Welburn, 2001). Furthermore, there is evidence that some *S. glossinidius* genotypes have a propensity to enhance the rates of establishment of certain trypanosome subspecies (Geiger et al., 2007). This suggests that tsetse flies, their bacterial symbionts, and the parasites they transmit have coevolved toward a mutually beneficial equilibrium.

Culturing facultative symbionts

Reduced metabolic plasticity

Most of the well-studied bacterial symbionts of multicellular organisms (e.g., the symbionts of soil nematodes, *Photorhabdus* and *Xenorhabdus* spp., or the Hawaiian Bobtail squid symbiont *Vibrio fischeri*) are maintained by the continual horizontal reinfection of their hosts (Forst et al., 1997; Ruby and Lee, 1998). During transmission, these symbionts are exposed to environments that are quite different from the ones provided by their mutualistic partners. In addition, the majority of bacteria undergoing transmission will not immediately reassociate with a susceptible host. Under these circumstances, one might expect that, independent of the coevolutionary processes that gave rise to the specificity of their mutualistic associations, frequent episodes of selection for the maintenance of genes required for this free-living stage should prevent these symbionts from becoming specialists. This idea is supported by the fact that these symbionts are readily cultured in the laboratory, and that *Photorhabdus* spp. (Bleakley and Chen, 1999; Gerrard et al., 2006; Weissfeld et al., 2005) and *V. fischeri* (Ruby and Lee, 1998) are found in environments where they are not associated with animal hosts.

Studies of host-symbiont coevolution show that many facultative symbionts undergo horizontal transfer during the early stages of their association with insects (Moran and Dunbar, 2006; Russell et al., 2003; Sandstrom et al., 2001; Thao and Baumann, 2004). However, over evolutionary time they switch to a predominantly vertical (maternal) mode of transmission (Dale and Moran, 2006). The adoption of a lifestyle virtually devoid of environmental changes renders obsolete many of the genes that were required for survival in the free-living stage. The relaxation of selection drives the accumulation of mutations and the loss of these genes from the symbiont genome (Akman et al., 2002; Andersson and Andersson, 1999; Moran and Wernegreen, 2000; Moran and Mira, 2001; van Ham et al., 2003). This process of genome degeneration or “streamlining” leads to the evolution of a highly specialized gene inventory. Although this is a fascinating evolutionary process, specialization ultimately undermines the study of these symbionts in the laboratory set-

ting, by imposing constraints on our ability to culture and manipulate these organisms. However, several facultative symbionts have now been isolated from their insect hosts and cultured in the laboratory (Dale et al., 2006; Dale and Maudlin, 1999; Darby et al., 2005; Hypsa and Dale, 1997; O'Neill et al., 1997).

Symbiont isolation

The first step in the cultivation of insect symbionts is the isolation of bacteria from host tissues. Facultative symbionts generally inhabit a wide range of host tissues, including insect hemolymph, and can exist both intracellularly and extracellularly (Chen and Purcell, 1997; Cheng and Aksoy, 1999; Fukatsu et al., 2000; Hypsa and Dale, 1997; Tsuchida et al., 2005). Thus, bacteria can usually be obtained either by aseptically collecting symbiont-rich hemolymph or by homogenizing whole insects in sterile medium. The relative simplicity of these procedures belies one of the major problems encountered in culturing attempts, namely contamination by foreign microorganisms. Because cultured insect symbionts have relatively slow growth rates, they are rapidly overwhelmed by contaminating microbes. Culturing attempts should therefore be made with large numbers of symbionts (i.e., a large initial inoculum), and special care must be taken to avoid the introduction of contaminating microorganisms during this initial isolation procedure. Insects must be surface-sterilized by chemical and/or physical means prior to symbiont collection. Because foreign microbes may be acquired through feeding, symbionts should also be isolated from insects on developmental stages that have not yet had the opportunity to feed (Pontes and Dale, 2006).

The potential for contamination can be further reduced by supplementing the symbiont growth medium with antibiotics that specifically target contaminating microbes. For example, given that insect symbionts are constantly exposed to the immune defenses of their hosts, they are expected to be naturally resistant to high concentrations of insect-derived antimicrobial compounds, such as cecropins and other cationic peptides (Dale et al., 2006; Gillespie et al., 1997; Haines et al., 2003). Commercially available versions of these compounds can therefore be used to inhibit growth of contaminating microbes that may be present in the insect material and/or introduced during symbiont collection (Dale et al., 2006).

Growth conditions

Once bacteria have been isolated from an insect host, they must be maintained in a culture medium that provides all of the nutrients that are necessary for growth. Because symbionts normally live in a stable and nutrient-rich host environment, they are expected to display reduced metabolic plasticity in comparison to free-living relatives. The genomic content and the level of specialization toward the host ultimately dictate the nutritional and environmental requirements of each symbiont. Given that these may vary among different symbionts, cultured insect cells are generally used as an initial platform in attempts to cultivate these bacteria (Dale and Maudlin, 1999; Darby et al., 2005; Hypsa and Dale, 1997; O'Neill et al., 1997). This is based on the rationale that these cell lines provide symbionts with conditions that are similar to those encountered in the natural environment. At the present time there are numerous insect cell lines available in laboratories, stock centers, and commercial repositories, but there is little known about compatibility between different cell lines and symbionts (Pontes and Dale, 2006).

The cultivation of insect symbionts within insect cell lines serves two major purposes. First, it provides a means to study the molecular and ecological basis of interactions between the symbiotic bacteria and the insect cells in the laboratory (Dale et al., 2001, 2006; Darby et al., 2005). Second, insect cell lines provide a means to rapidly propagate large numbers of symbiotic bacteria in the laboratory (Dale et al., 2006). Bacterial cells can be recovered with high purity from infected cell cultures by differential centrifugation, providing a source of live material that would be extremely difficult to obtain directly from insects. The large numbers of symbionts recovered from these cultures can be used in a wide range of downstream experiments including whole genome sequencing and host microinjection (Chen and Purcell, 1997; Montllor et al., 2002; Oliver et al., 2003, 2005; Scarborough et al., 2005). Insect cell lines also provide a platform for the development of axenic (cell-free) culture systems. Indeed, the culture medium used to propagate the cell line often serves as a base for the development of media formulations for axenic culture.

It stands to reason that a larger array of experimental manipulations can be made, and consequently greater biological insight can be gained if symbionts can also be maintained in pure culture. Because the symbionts are notoriously fastidious, the establishment of axenic cultures presents a number of challenges. The loss of metabolic plasticity due to specialization to the host environment is reflected in the symbionts' complex nutritional requirements. Defined media formulations have been developed for the axenic cultivation of some facultative symbionts. However, this type of knowledge is lacking for symbionts that have not yet been cultured axenically and studied in the laboratory. Therefore, axenic culturing attempts are generally initiated with complex media formulations that are designed to support the growth of insect cells. Because symbionts live within insects and can often be cultivated in insect cell lines, it is logical to assume that the nutritional requirements of the insect cell are similar to those of the symbiont—with the caveat that the insect cell might biosynthesize one or more essential symbiont nutrients.

In addition to nutrients, other environmental factors need to be taken into consideration in culture attempts. In view of the long-term association of symbionts with their insect hosts, it is logical to assume that symbionts are impaired in their ability to deal with environmental stress. This is because symbionts live in a protected niche, in which their host is anticipated to provide protection against environmental perturbances and stress (including oxidative stress, physiological stress, and heat shock). Given these circumstances one would expect symbionts to lose the ability to counteract stress as a result of the relaxed selection imposed in the symbiotic lifestyle. Not surprisingly, the two facultative symbionts that have so far been isolated in pure culture are microaerophilic, which means that they cannot tolerate atmospheric levels of oxygen (Dale et al., 2006; Dale and Maudlin, 1999). Although this is likely a common feature resulting from adaptation to a life within an insect, it creates another major difficulty in culturing these bacteria under axenic conditions. In the absence of insect cells, symbionts are not able to survive the assault of reactive oxygen species (ROS) present in the culture medium. The establishment of pure symbiont cultures may therefore require large initial inoculums (to effectively reduce the ratio of bacterial cells to ROS) and/or the supplementation of culture media with enzymes (e.g., catalases, superoxide dismutases, reducing agents) that detoxify ROS.

The axenic cultivation of symbionts is generally accomplished in liquid media. However, given the need to isolate clonal lineages, the development of plate cultures is of great importance. Solid phase culture systems have been developed for *S. glossinidius* and *Ca. Arsenophonus arthropodicus* (Dale et al., 2006; Dale and Maudlin, 1999). These cultures can be established by plating the symbionts on solid media containing 0.7%–1% agar (w/v) and allowing them to grow for 5 to 7 days, under microaerophilic conditions (5% oxygen,

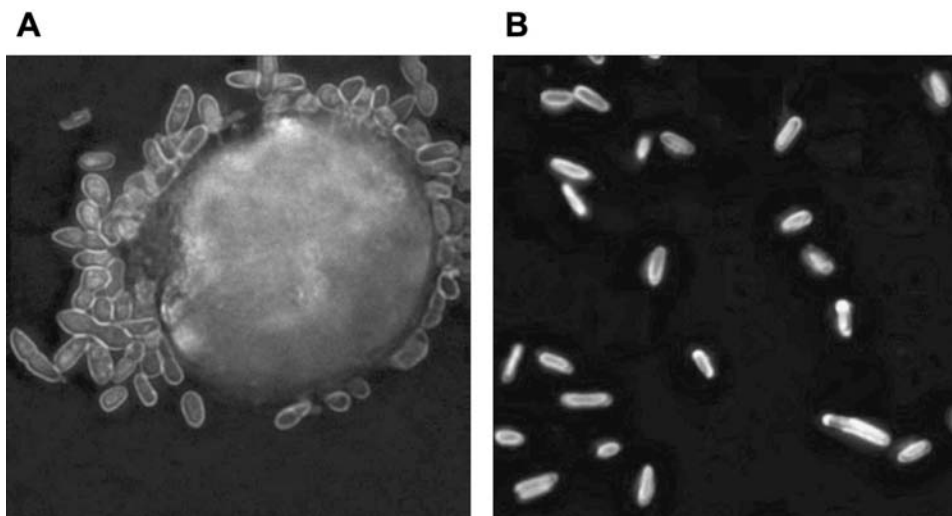


Figure 18.2 (Color figure follows p. 238.) Symbionts in insect cell culture and pure culture. Insect cell lines are useful for the culture of facultative symbionts and the study of interactions between symbionts and host cells. In plate A, *Ca. Arsenophonus arthropodicus* is attached to the surface of an *Aedes albopictus* C6/36 cell. The insect cell and bacteria were fixed and stained with FM4-64 (which binds to cellular lipids) and DAPI (which binds to nucleic acids) and visualized by deconvolution fluorescence microscopy. In plate B, live bacterial cells from a pure culture of *S. glossinidius* were visualized by fluorescence microscopy following staining with FM4-64 and DAPI. Pure culture isolation provides opportunities for the genetic manipulation of facultative symbionts.

10% carbon dioxide, and 85% nitrogen). The establishment of plate cultures, however, is not as straightforward as it seems. The main difficulty in this procedure arises, again, from oxidative stress imposed on the symbionts during plating under aerobic conditions. Preventive measures must therefore be taken to minimize the deleterious effects of ROS. Exogenous catalase or fresh vertebrate blood (an excellent source of catalase, superoxide dismutase, and other enzymes involved in the oxidative stress response) can be incorporated into solid media to reduce the effects of ROS. Not surprisingly, when these symbionts are spread on plates containing these supplements they display an increase in plating efficiency and growth rate (Dale and Maudlin, 1999; Matthew et al., 2005).

Maintenance of cultures

Microscopy provides a basis for the general examination of both insect cell lines and axenic cultures (Figure 18.2). However, because cultures of insect symbionts are prone to contamination, their integrity needs constant evaluation. Two methods are generally used for this purpose. In the case of mixed cultures with insect cells and axenic liquid cultures, symbionts and contaminants can be detected using *in situ* hybridization with universal and symbiont-specific probes (Darby et al., 2005; Fukatsu et al., 1998). On the other hand, if the symbiont can be cultivated on solid media, a simpler procedure can be used. Following plating, the identity of a single colony can be determined by PCR with specific primers. Because colonies are each derived from a single cell, they can be used to initiate fresh monoclonal cultures.

When bacterial pathogens are maintained in the laboratory for a long period of time, they often attenuate their virulence toward the natural host. This is due to the relaxation of selection on those bacterial genes that facilitate essential interactions between the pathogen and host. It stands to reason that insect symbiont cultures will suffer the same fate if they are also maintained in the laboratory for long periods of time. Thus, it is prudent to implement long-term storage of symbiont cells shortly after they are established in culture. Stocks of symbiont cells can then be recovered from storage at regular intervals to ensure the genetic integrity of strains used in experiments. Fortunately, these bacteria can be stored over long periods of time in 25% (w/v) glycerol stocks at -80°C .

Genetic modification of facultative symbionts

Goals and basic approaches

In the context of insect symbionts, two basic goals can be met through the application of recombinant DNA technology. First, the target organism can be modified to carry and (optionally) exhibit controlled expression of a gene of interest. Second, the genome of the target organism can be modified to change the expression of a gene that is already in residence. This includes disrupting or enhancing the timing or level of expression of a resident gene. In this section we describe a number of experimental approaches that should have broad applicability in symbionts that are allied to the gamma subdivision of Proteobacteria. These methods have proven effective for *S. glossinidius* (Dale and Maudlin, 1999) and *Ca. Arsenophonus arthropodicus* (Dale et al., 2006), which represent model systems in our laboratory. It should be noted that all of the techniques described are dependent on the ability to isolate recombinant clones in the form of single colonies.

Plasmid transformation

Plasmid transformation is perhaps the most basic and robust technique available for the manipulation of the bacterial genome. The successful transformation of insect symbionts with plasmids (Beard et al., 1993; Durvasula et al., 1997) was heralded with great excitement because it was seen as the first step in the development of pseudo-transgenic insects. However, it should be noted that plasmids have their pitfalls; the biggest problem is their lack of stability in the absence of selection. Thus, in reality, chromosomal transformations (discussed later) provide a more robust means for inducing permanent changes in the bacterial genome. But, in order to utilize these more complex techniques it is often necessary to introduce genetic constructs into bacteria in the form of plasmids.

Bacteria from many different phylogenetic groups have been successfully transformed with plasmids and these include insect symbionts (Beard et al., 1993; Dale et al., 2001, 2006). The basic requirement is to find a plasmid that is compatible with the strain of interest and for this reason plasmids have historically been classified into incompatibility groups. Fortunately, there are some plasmids that are known to be compatible with a wide range of bacterial phyla. These include plasmids from incompatibility group Q (Rawlings and Tietze, 2001), which have been used to transform *S. glossinidius* and *Ca. Arsenophonus arthropodicus* with high efficiency and stability (Dale et al., 2005).

Most cultured representatives of the Proteobacteria seem to be amenable to both chemical transformation and electroporation. In the case of *S. glossinidius* and *Ca. Arsenophonus arthropodicus*, both of these methods have proven successful. However, it should be noted that even under optimum conditions the transformation efficiencies (based on the number

of recombinant colonies recovered per μg plasmid DNA) obtained with these symbionts are typically 10- to 1000-fold lower than transformation efficiencies observed when the same plasmid is introduced into *Escherichia coli*. This is attributed in large part to oxidative stress encountered during the plating of bacterial cells, following transformation and recovery. Both *S. glossinidius* and *Ca. Arsenophonus arthropodicus* are most vulnerable to oxidative shock when they are spread at low density across the surface of an agar plate. Thus, care needs to be taken throughout the transformation procedure to ensure that exposure to oxygen is minimized.

Transposon mutagenesis

Transposon mutagenesis provides a means to disrupt or modulate existing genes in the symbiont genome. It is important to note that in this approach random mutations are introduced by the mobilization of transposable elements. The utility of this approach is therefore dependent on the implementation of screens that can be used to identify or select mutants that have a specific phenotype. The genetic changes responsible for the mutant phenotype can then be identified using the myriad techniques of bacterial genetics. As an example, the transposon Tn5 was used in the mutagenesis of *S. glossinidius* to generate mutant symbionts that were incapable of invading insect cells. The requisite mutants were selected simply by enriching for bacteria that were unable to penetrate insect cells *in vitro*. Following pure culture isolation of mutants, Tn5 insertions were identified in the *S. glossinidius* genome by cloning and sequencing (Dale et al., 2001).

Although a number of different transposable elements have been used to perform random mutagenesis in bacteria, Tn5 remains attractive because of its broad host range, insertion frequency, and ability to transpose randomly into target DNA (Reznikoff et al., 2004). Traditionally, Tn5 mutagenesis is performed using suicide plasmid constructs that provide Tn5 ends flanking a marker gene (e.g., an antibiotic resistance cassette). The Tn5 transposition functions are provided either in a distinct location on the same plasmid or on a different plasmid. When transposition functions are induced the Tn5 construct with the marker gene is transposed randomly into another location, resulting in the inactivation of target genes. The loss of the plasmid carrying the transposition functions prevents any further mobilization of the element. The efficiency of Tn5 transposition is known to vary considerably among species and strains of bacteria. This is largely dependent on the ability of the strain of interest to efficiently express the transposase protein *in vivo*. To address this issue, researchers developed a Tn5 transposition technique that involves the use of released Tn5 transposition complexes (Goryshin et al., 2000). These transposase-DNA complexes are electroporated directly into the bacterial cell to effect transposition and are reported to work with a wide range of bacterial species with high efficiency. We have used them successfully in *S. glossinidius* and *Ca. Arsenophonus arthropodicus* (Dale, unpublished observations).

Chemical mutagenesis and "TILLING"

Prior to the implementation of more sophisticated genetic techniques, mutagenesis was often performed using simple chemical mutagens including N-methyl-N'-nitro-N-nitrosoguanidine (MMNG) and ethyl methane sulfonate (EMS). The basic approach involves exposing a population of cells to a controlled dose of mutagen, to generate progeny that carry random genetic mutations. The application of this technique has traditionally been limited by two important considerations. First, it often proves difficult to strike a balance

between efficiency and selectivity; exposure to the mutagen must be carefully controlled in order to maximize the frequency of single mutations. Second, it should be noted that chemical mutagenesis is random; the experimenter needs to select or identify mutants of interest from a large pool of random mutants.

For more detailed information on the background and procedures involved in chemical mutagenesis, we refer the reader to Miller (1992). The basic protocols that have been described for *E. coli* can easily be adapted for other bacteria, including insect symbionts. Because various species and strains of bacteria are known to respond in different ways to mutagens and treatment regimes, it is of great importance to establish an experimental approach that yields an optimal level of mutagenesis and survival for a given strain. Mutagenesis is typically quantified by measuring the conversion of the wild-type strain to a readily identifiable mutant phenotype. Typically, we measure conversion to rifampicin and ciprofloxacin resistance, mediated by mutations in the RNA polymerase β -subunit (RpoB) and DNA gyrase (GyrA) enzymes respectively (Vila et al., 1994; Xu et al., 2005). Using both selectable markers, it is possible to determine the frequencies of both single and double mutants to more accurately optimize the dose of mutagen.

The major challenge associated with the use of reverse genetic strategies lies in the identification or characterization of mutants that have no obvious, selectable phenotype. Fortunately, high-throughput methods have recently been developed to ameliorate this problem. TILLING (Targeting Induced Local Lesions in Genomes) is one such technique that has been developed to use a mismatch-specific enzyme to identify mutations in a gene of interest through high-throughput heteroduplex analysis (Till et al., 2003). Although the basic technique has been developed for use with higher organisms, it can readily be adapted for use with bacteria, as outlined in Figure 18.3.

Allelic exchange and recombineering

Reverse genetics approaches allow the investigator to mutagenize a specific target gene to investigate gene or protein function. Frequently, this involves engineering the replacement of a wild-type sequence with a cloned mutant allele. Although several techniques have been described to facilitate allelic exchange, all are reliant upon the process of homologous recombination. Thus, the investigator introduces cloned DNA into the target cell and the cellular recombination systems are expected to integrate this DNA into the target cell genome. For example, one might construct a recombinant DNA fragment consisting of an antibiotic resistance cassette flanked by DNA sequences that share homology with a gene of interest in the target organism. If the target organism recombines the DNA fragment within its own chromosome, this should effect the replacement (knockout) of the target gene with a selectable antibiotic resistance cassette.

Although allelic exchange has been used to effect gene replacements in a wide range of organisms, there are many factors known to influence the efficiency of the recombination process in different species and strains of bacteria. Although we have successfully used allelic exchange to effect gene knockout in *S. glossinidius* (Dale et al., 2005), numerous failed attempts indicate that, overall, the technique has very low efficiency. Notably, many insect symbionts are known to lack essential genic components of DNA recombination and repair pathways, presumably as a result of relaxed selection imposed by the symbiotic lifestyle (Moran and Wernegreen, 2000). Although *S. glossinidius* does maintain a full complement of the *rec* recombination/repair genes, the activities of the resulting enzymes are likely to be diminished in comparison to free-living bacteria.

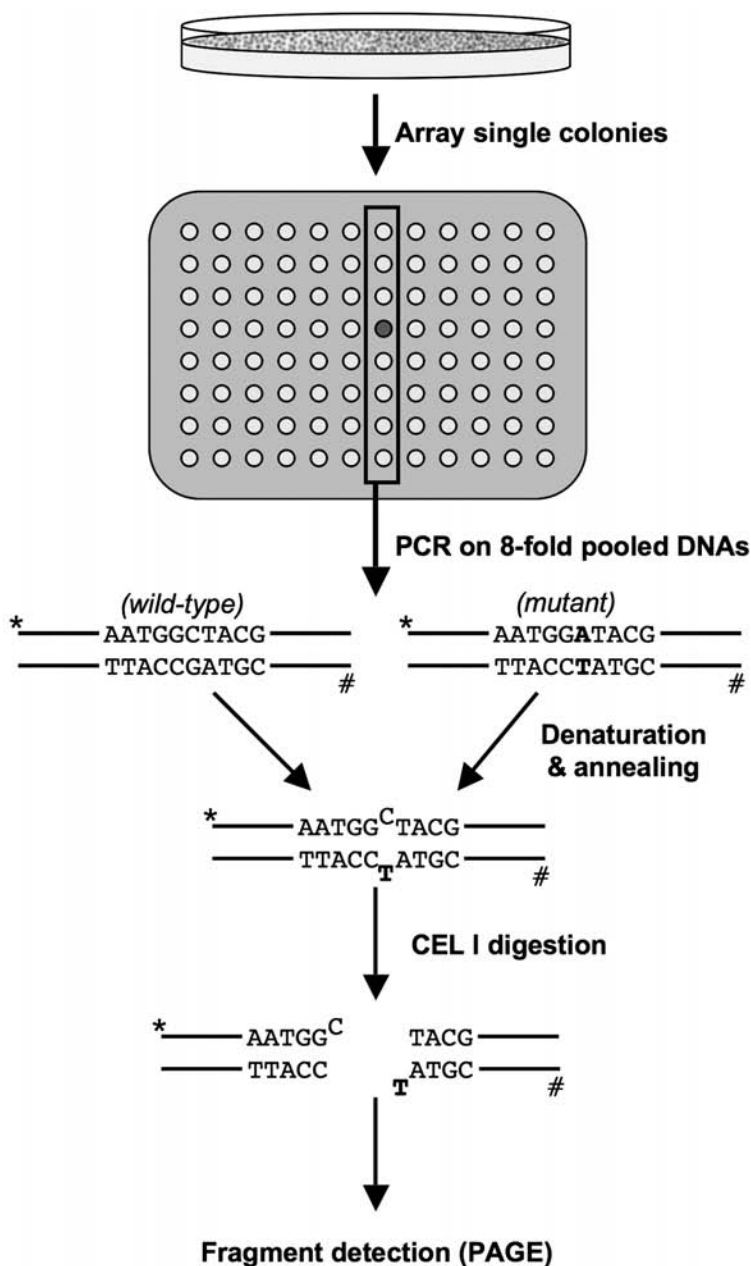


Figure 18.3 TILLING for mutations in bacterial genomes. Following chemical mutagenesis, clones (colonies) are arrayed into 96-well plates for archiving and screening. Genomic DNA is prepared from pools of eight clones to decrease screening time. PCR is performed on pooled DNA using target gene specific primers labeled with different fluorophores (designated by asterisk and hash symbols). PCR products are then denatured and reannealed to allow heteroduplex products to form in the presence of wild-type and mutant DNA. Resulting DNAs are then digested with celery extract endonuclease (CEL I) to cleave double stranded DNA at mismatched bases. Truncated cleavage products are then detected on denaturing polyacrylamide gels with a LICOR 4300 analysis system. Individual mutant clones are then isolated from positive pools by PCR amplification and sequencing of target genes.

Datsenko and Wanner (2000) recently described an approach (dubbed “recombineering”) that utilizes exogenous recombination enzymes to catalyze chromosomal gene replacements through allelic exchange (Court et al., 2002). The “recombineering” approach uses bacteriophage λ Red recombination functions and therefore should be particularly useful in studies focusing on bacterial strains that have limited or defective DNA recombination enzymes, including many facultative symbionts. The technique has broad applicability in the context of bacterial genetic engineering and is particularly useful for the construction of null (knockout) mutants. To utilize the “recombineering” approach, the target organism must first be transformed with a plasmid that maintains the λ Red recombination functions under the control of an inducible arabinose promoter. The resulting strain is then secondarily transformed with a linear DNA fragment that shares homology with a chromosomal sequence that it is designed to replace. If the experimenter wishes to engineer a null mutant, the linear DNA fragment would typically consist of a selectable antibiotic-resistance cassette flanked by sequences that share homology with the chromosomal recombination target. In order to stimulate recombination, the λ Red functions are activated by arabinose induction several hours prior to the introduction of the linear DNA fragment. The active λ Red recombination enzymes are then expected to catalyze homologous recombination between the linear DNA fragment and the chromosomal target sequence, producing double-crossover mutants that can readily be identified by antibiotic selection. Once mutants have been identified, the λ Red plasmid is typically removed (cured) by heat treatment to ensure that no further λ Red-mediated recombination occurs. Once the double-crossover mutant has been verified by selection and sequencing, the chromosomal antibiotic resistance cassette can be removed by FLP-mediated recombination, to yield a mutant with a minimal null (knockout) allele.

Conclusions

Advances in genomics, molecular biology techniques, and microbiological methods provide new opportunities to advance our understanding of the associations involving insects and facultative symbionts. Based on the limited amount of knowledge gained to date it is clear that facultative symbionts play important roles in their host insects, shaping their ecology, evolution, and interactions with other forms of life on Earth. Furthermore, many of the insects that harbor facultative symbionts are classified as pests of medical, veterinary, and agricultural importance. Therefore, it seems likely that the study of facultative symbionts will provide new opportunities for the control of pest insects and vector-borne diseases. Over the course of the 20th century, mainstream microbiology has focused almost exclusively on the study of pathogenic bacteria, with the principal aim of increasing our understanding of the mechanisms of disease. Recent studies in the field of symbiosis show that many mutualistic symbionts utilize the same molecular mechanisms as pathogens to facilitate their interactions with host cells and tissues. Further studies are therefore needed to provide a more complete understanding of the ecology, evolutionary history, and mechanisms of animal–bacterial associations.

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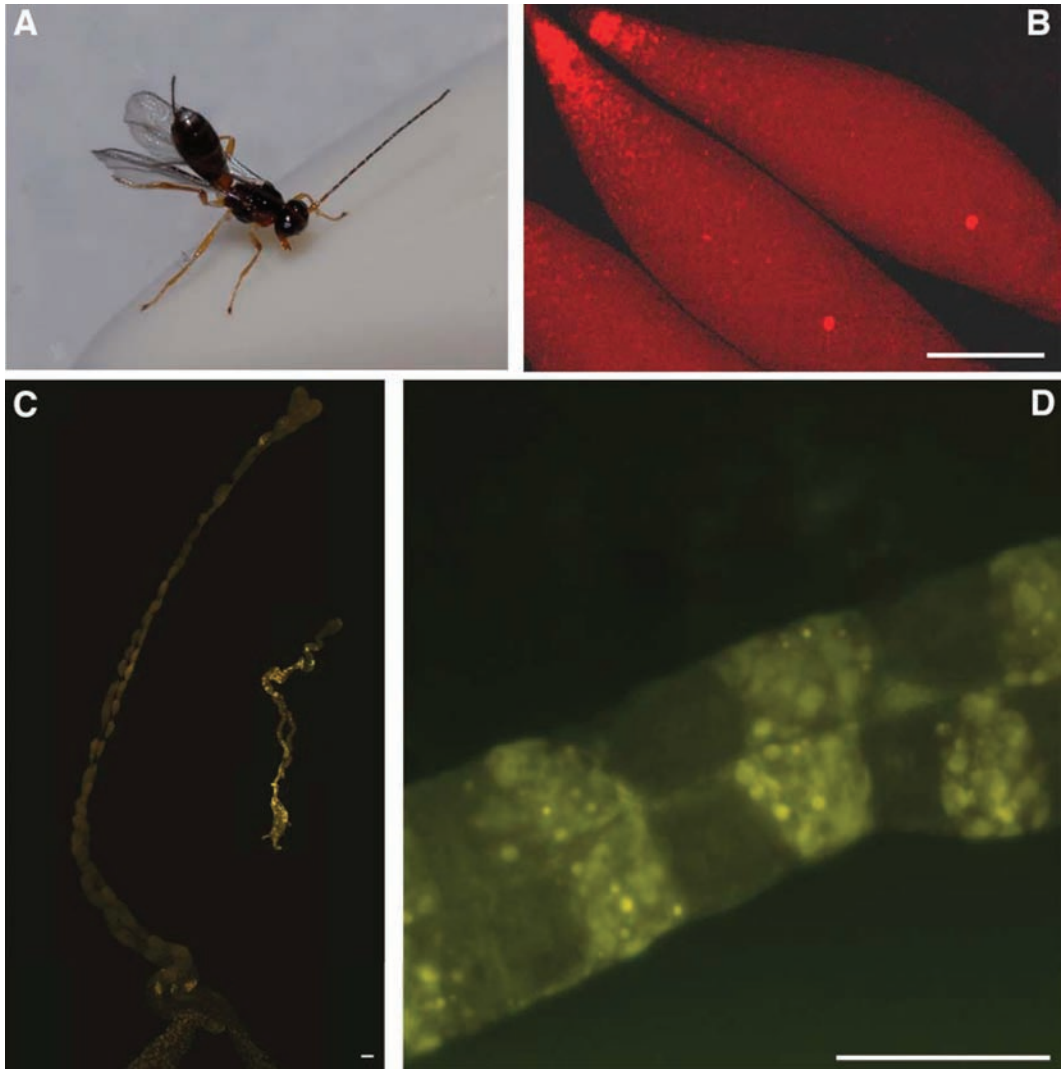
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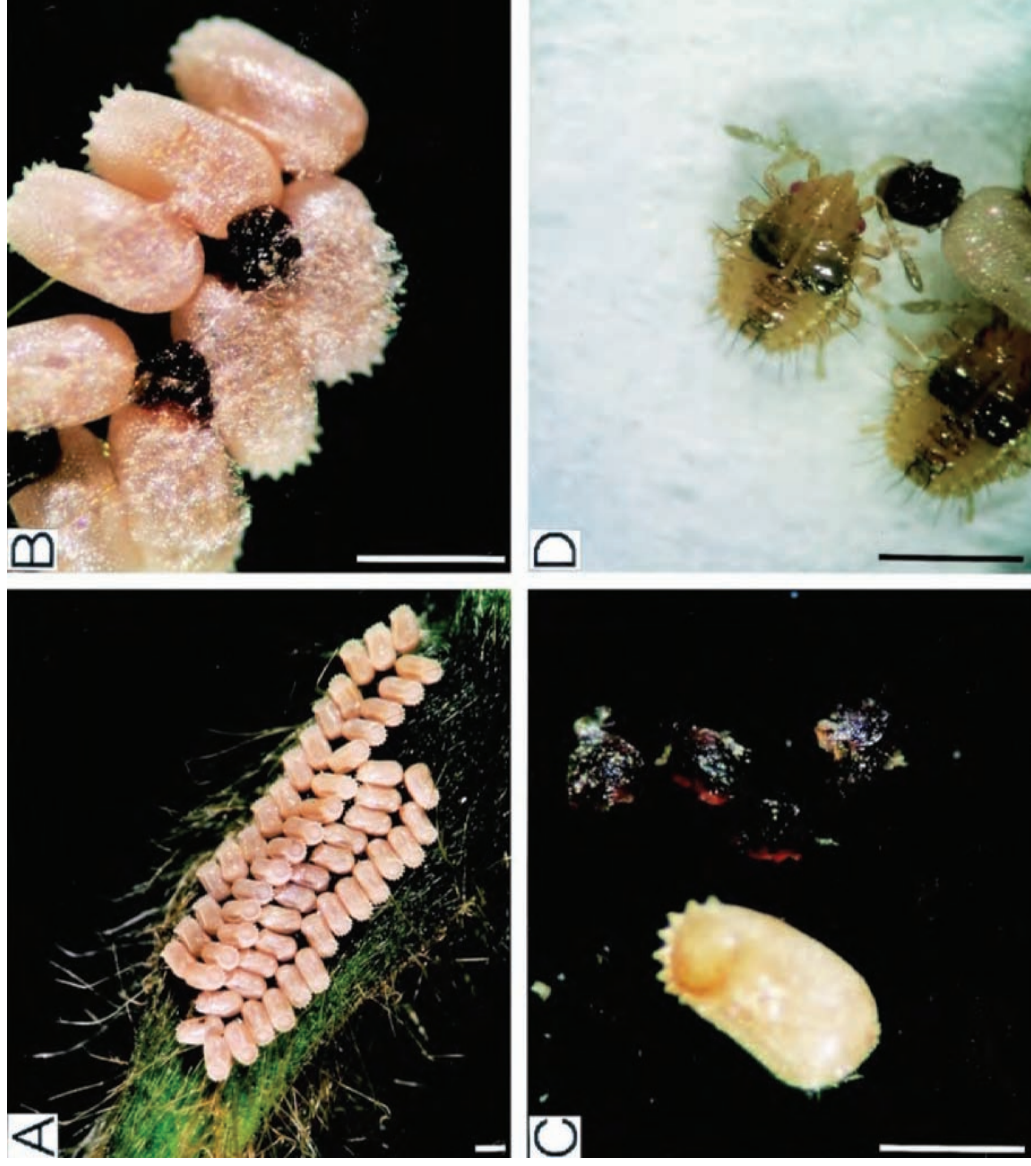
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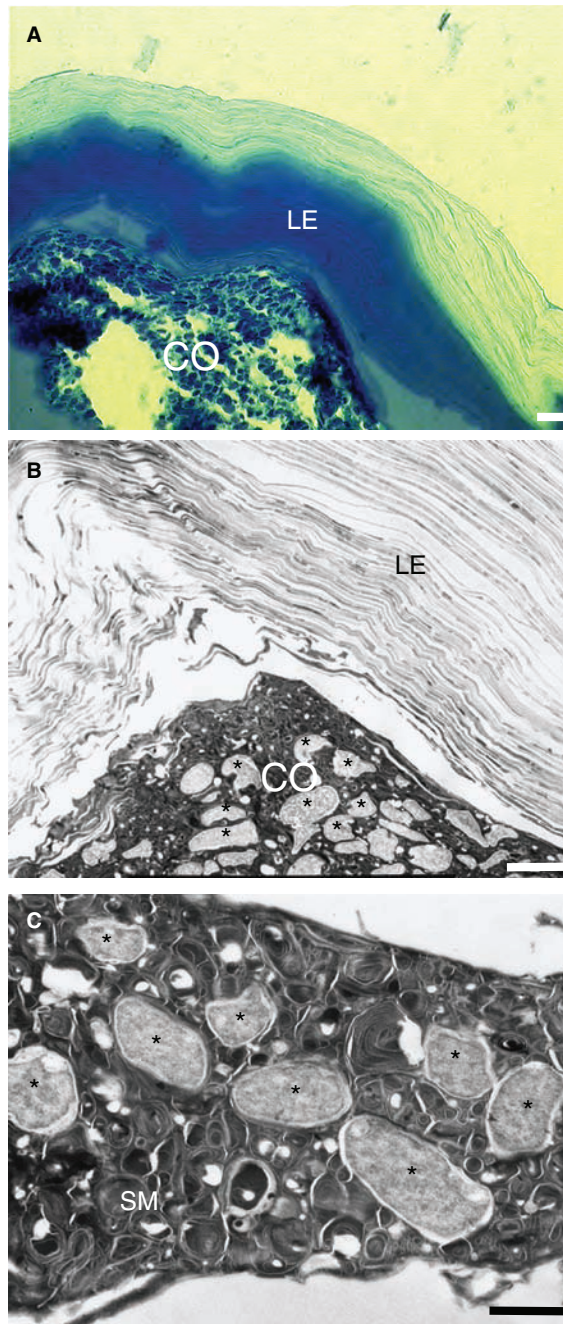
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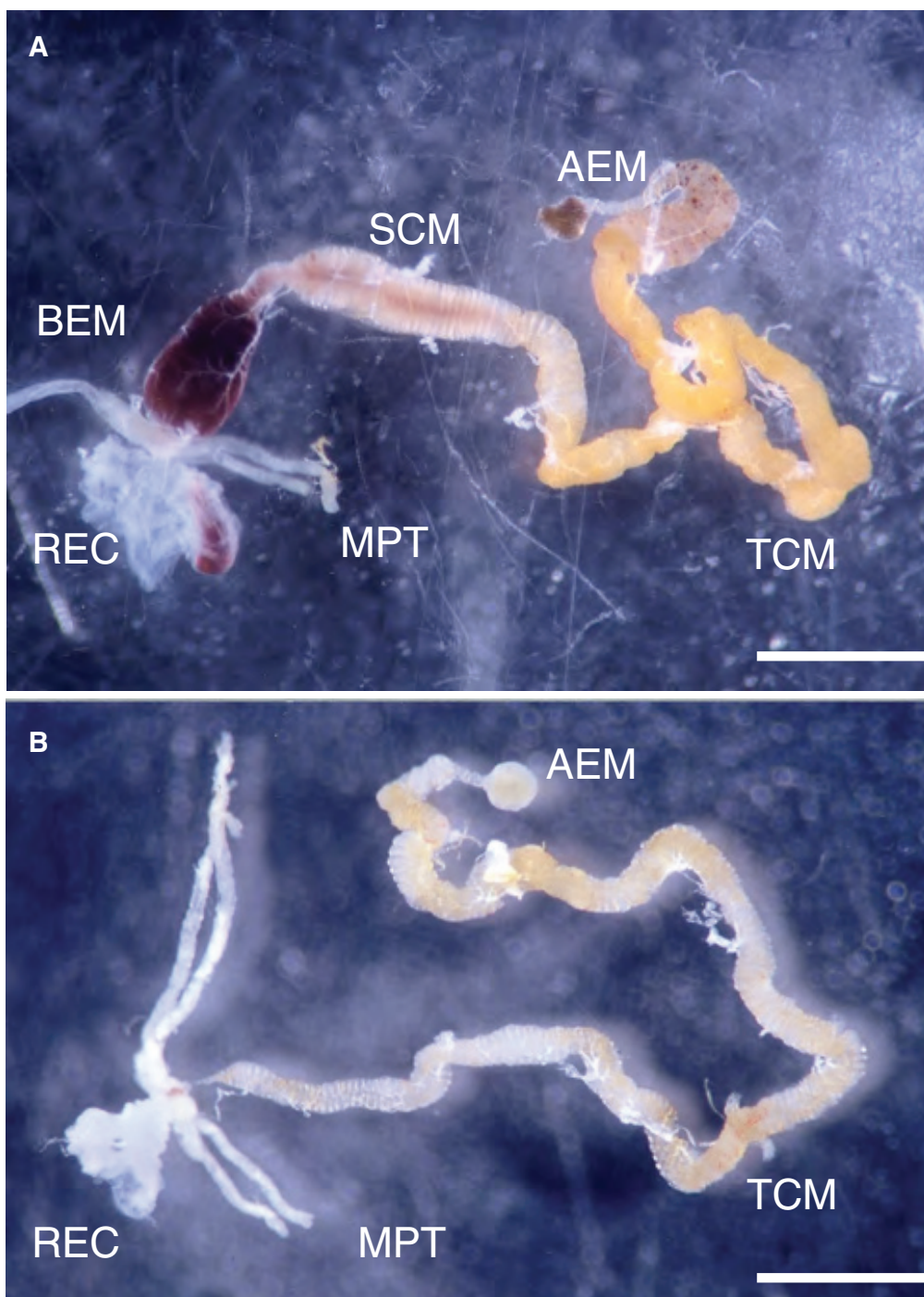
Color Figure 3.2 The role of PCD in the interaction between *Wolbachia* and *Asobara tabida*. (A) An *A. tabida* female. (Picture from FV and F. Debias.) (B) Oocytes of *A. tabida* infected with *Wolbachia*. Bacteria appear clustered at the posterior end of the oocyte (at the top of the picture). Scale bar = 50 μ m. (Picture adapted from Dedeine et al., PNAS, 2001.) (C) Ovaries from infected (left) and uninfected (right) females. Note the difference in the size of the organ when the symbiont is removed. Scale bar = 50 μ m. (Picture adapted from Pannebakker et al., PNAS, 2007.) (D) Part of the ovary of an uninfected *A. tabida* female stained with TUNEL. Staining appears specific to the nurse cells of the egg chamber while no PCD is detected within the oocytes. Scale bar = 50 μ m. (Picture from BAP)



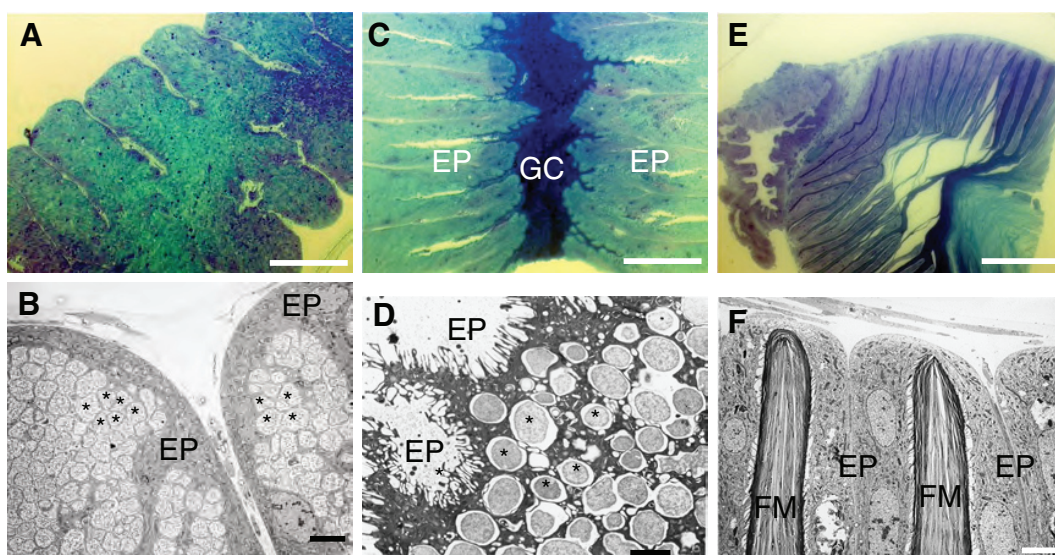
Color Figure 5.3 (A) Egg masses of *Megacopta punctatissima* laid on a bud of *Pueraria lobata*. (B) Symbiont capsules, dark brown in color, placed on the underside of an egg mass. (C) An isolated egg and capsules. (D) A newborn nymph probing a symbiont capsule. Bars show 0.5 mm. (From Fukatsu, T., and Hosokawa, T. [2002]. *Appl. Environ. Microbiol.* 68: 389–396. With permission.)



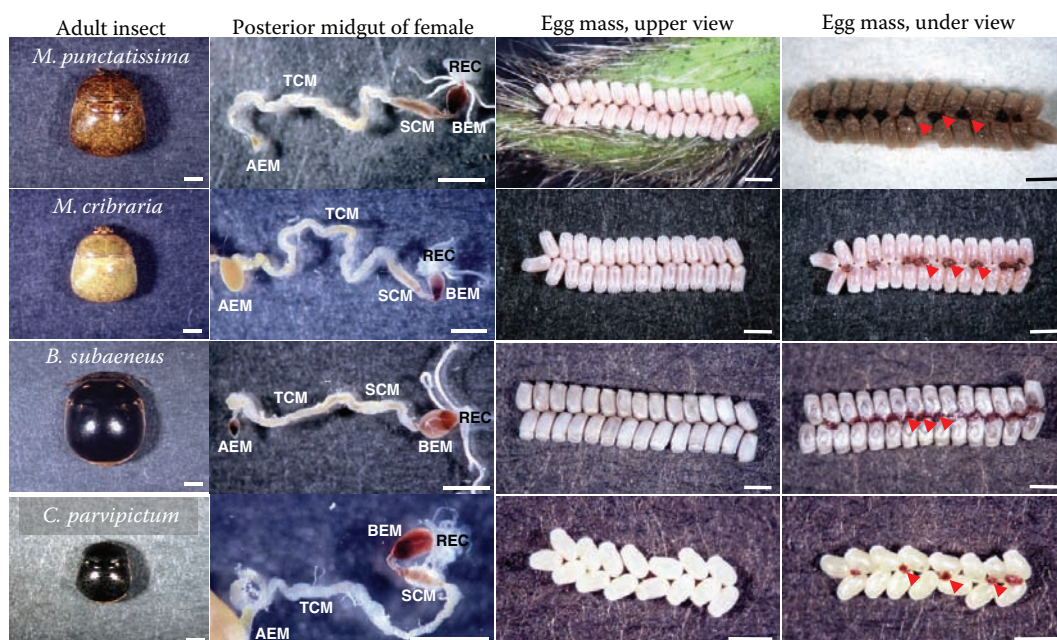
Color Figure 5.4 Light and electron microscopy of sectioned symbiont capsules of *Megacopta punctatissima*. (A) Light microscopic image of a symbiont capsule. (B) Electron microscopic image of a symbiont capsule. Symbiont cells are shown by asterisks. (C) Electron microscopic image of the capsule content. Abbreviations: CO, capsule content; LE, layered envelope; SM, secretion matrix. Bars show 2 μm in (A) and (B), and 1 μm in (C). (From Hosokawa, T., Kikuchi, Y., Meng, X.Y., and Fukatsu, T. [2005]. *FEMS Microbiol. Ecol.* **54**: 471–477. With permission.)



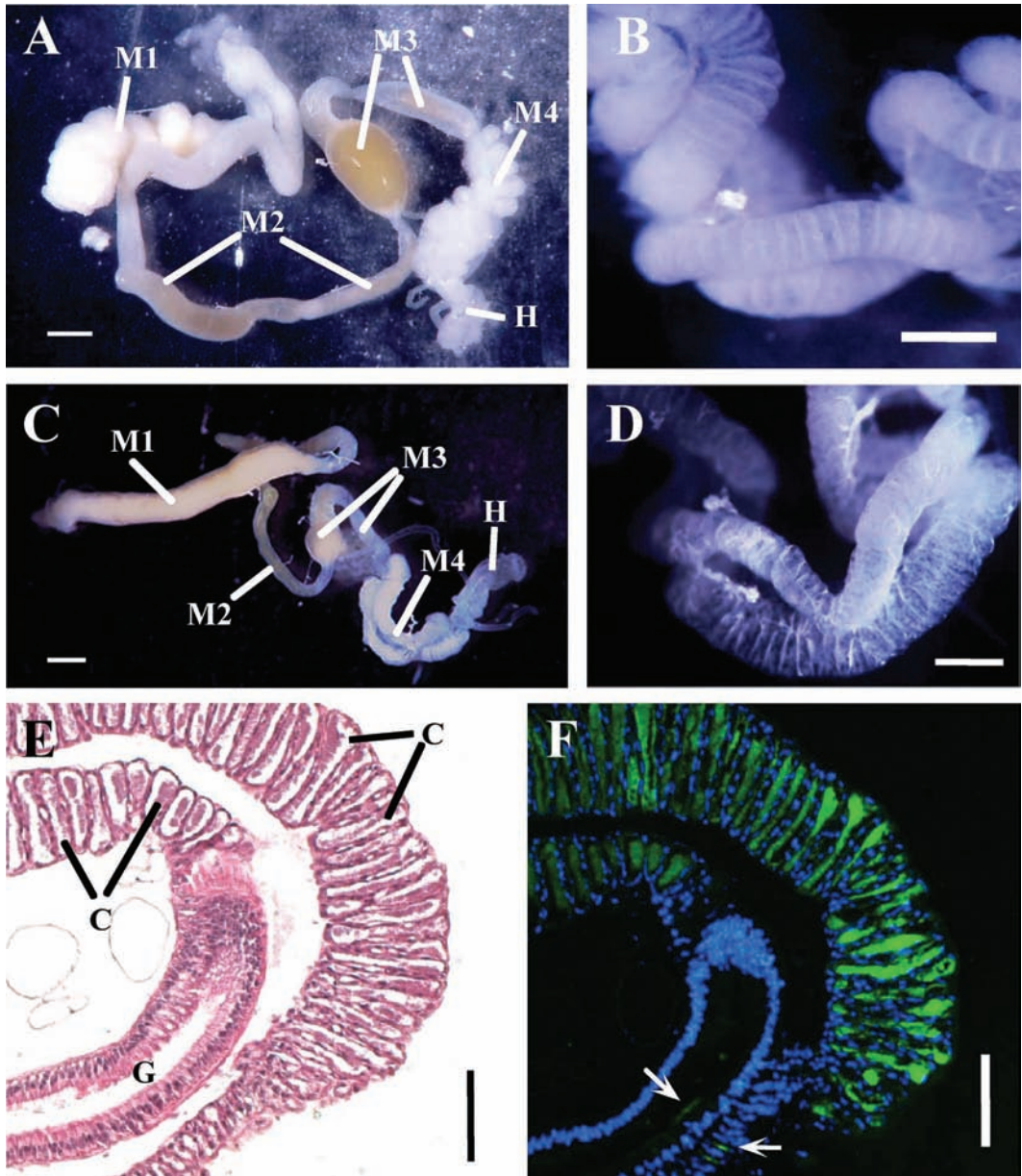
Color Figure 5.5 Dissected posterior midgut from adult female (A) and adult male (B) of *Megacopta punctatissima*. Abbreviations: AEM, anterior enlarged midgut section; BEM, brownish enlarged midgut end section; MPT, Malpighian tubules; REC, rectum; SCM, swollen crypt-bearing midgut section; TCM, thin crypt-bearing midgut section. Bars show 1 mm. (From Hosokawa, T., Kikuchi, Y., Meng, X.Y., and Fukatsu T. [2005]. *FEMS Microbiol. Ecol.* **54**: 471–477. With permission.)



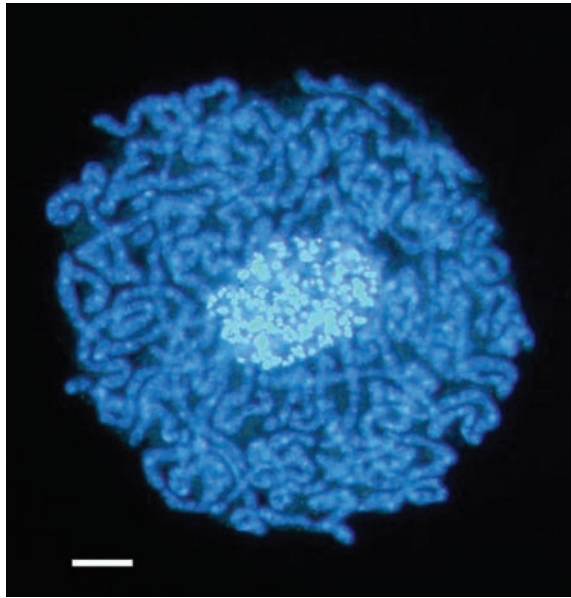
Color Figure 5.6 Light and electron microscopy of the midgut sections of *Megacopta punctatissima*. (A) Thin crypt-bearing midgut section (TCM). (B) Crypts of TCM, where numerous symbiont cells (asterisks) and thin epithelium are seen. (C) Swollen crypt-bearing midgut section (SCM). (D) Crypt of SCM, where the matrix is secreted. In the main tract of the midgut, a number of symbiont cells (asterisks) are embedded in the matrix. (E) Brownish enlarged midgut end section (BEM). (F) Crypts of BEM, whose cavity is filled with filament-like materials of the capsule envelope. Abbreviations: EP, epithelium; FM, filament-like material; GC, gut content. Bars show 50 μm in (A), (C), and (E), and 2 μm in (B), (D), and (F). (From Hosokawa, T., Kikuchi, Y., Meng, X.Y., and Fukatsu T. [2005]. *FEMS Microbiol. Ecol.* **54**: 471–477. With permission.)



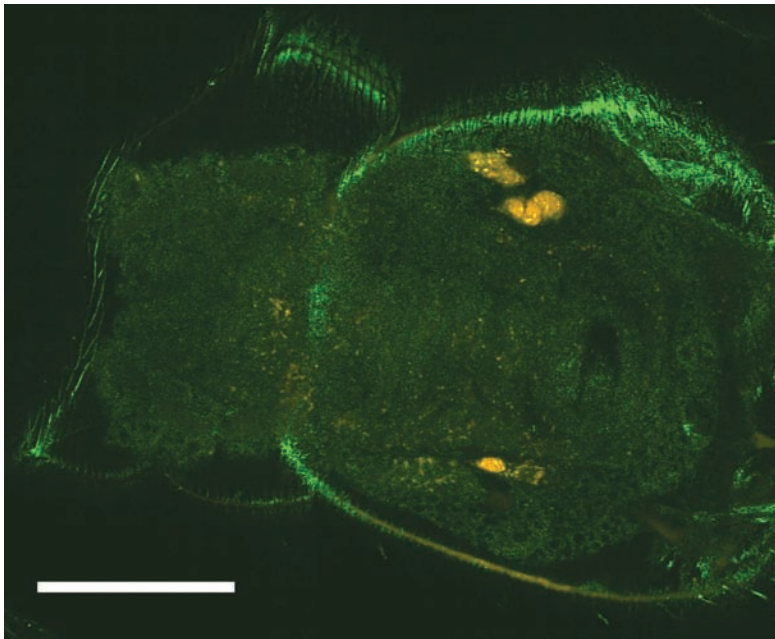
Color Figure 5.8 Four representative species of Japanese plataspid stinkbugs, their posterior midgut with capsule-producing organs, and their egg masses with symbiont capsules.



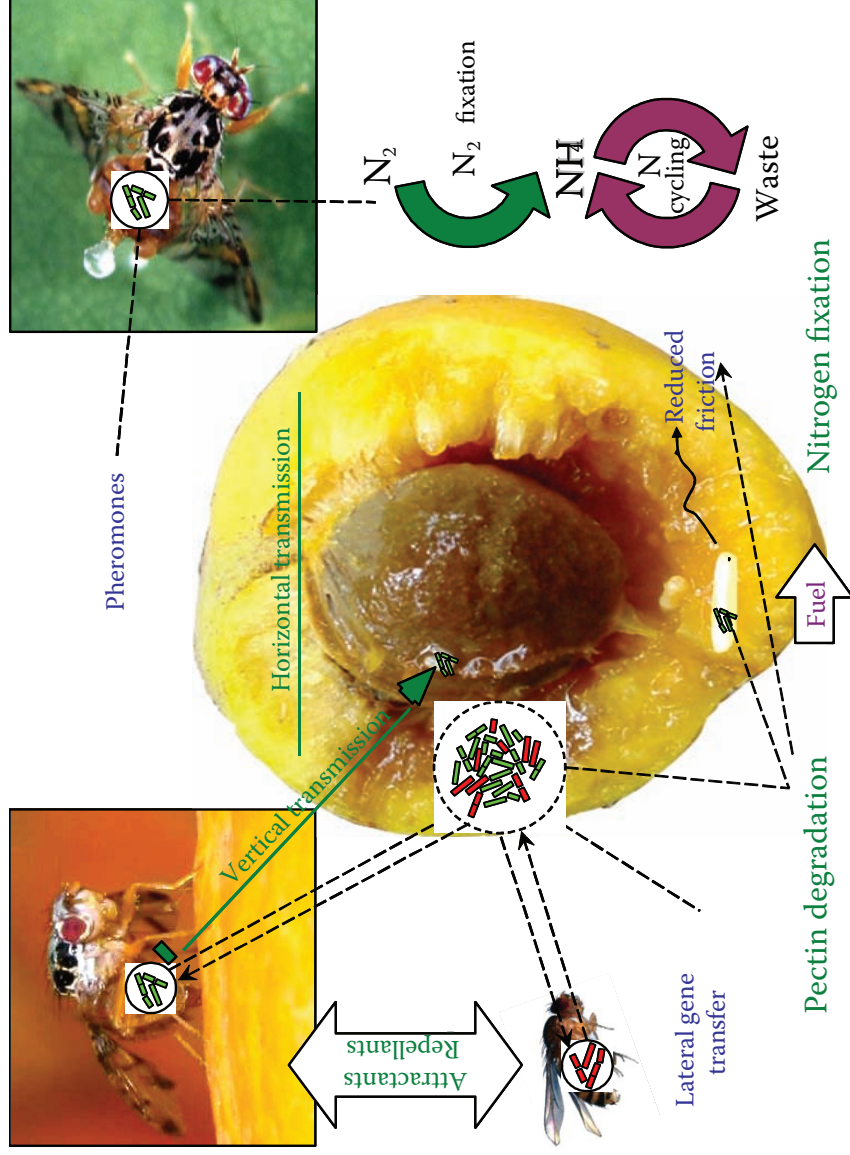
Color Figure 7.2 Midgut organization of *R. pedestris* and *L. chinensis*. A dissected midgut of (A) *R. pedestris* and (C) *L. chinensis*. Enlarged image of midgut fourth section with crypts of (B) *R. pedestris* and (D) *L. chinensis*. (E) A tissue section of midgut crypts of *R. pedestris*, stained with hematoxylin and eosin. (F) Fluorescent *in situ* hybridization of a tissue section of midgut crypts of *R. pedestris*, targeting 16S rRNA of the *Burkholderia* symbiont. Green signals are due to the *Burkholderia*-specific probe Cy3-Alsymb16S. Blue signals are nuclei of the host cells visualized by DAPI. Arrows indicate the *Burkholderia* signals in the main tract of the midgut and those in the ducts connecting the crypts with the main tract. Bars, 0.5 mm in (A) and (B), 0.2 mm in (C) and (D), and 100 μ m in (E) and (F). Abbreviations: C, crypt; G, gut; M1, midgut first section; M2, midgut second section; M3, midgut third section; M4, midgut fourth section (symbiotic organ); H, hindgut. (Modified from Kikuchi, Y., Meng, X.Y., and Fukatsu, T. [2005]. *Appl. Environ. Microbiol.* 71: 4035–4043. With permission.)



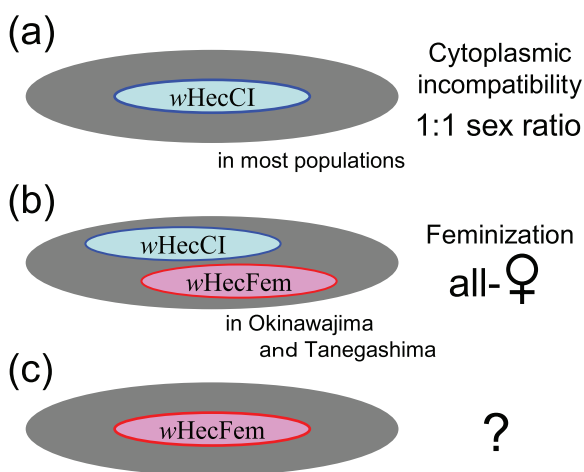
Color Figure 8.1 The bacteriocyte of *P. venusta*. Tubular cells surrounding the host nucleus (center) are *Carsonella*. Bar = 10 μm . (Modified from Nakabachi, A., Yamashita, A., Toh, H., Ishikawa, H., Dunbar, H.E., Moran, N.A., and Hattori, M. [2006]. *Science* **314**: 267.)



Color Figure 10.1 *Rickettsia* in a paired mycetome at both sides of the body between ovaries and midgut in a teneral of the booklouse *L. bostrychophila* (Psocoptera). Ventral view of a horizontal section. Extracellular *Rickettsia* are visible in the hemolymph as well as *Rickettsia*-carrying mycetocytes in other tissues. The mycetome on the left side of the animal is duplicated. Confocal microscopy picture with a *Rickettsia*-specific probe (yellow channel). Bar 100 μm . (Modified from Perotti, M.A., Clarke, H.K., Turner, B.D., and Braig, H.R. [2006]. *Rickettsia* as obligate and mycetomic bacteria. *FASEB J.* **20**: 2372–2374 and E1646–E1656. With permission from FASEB.)

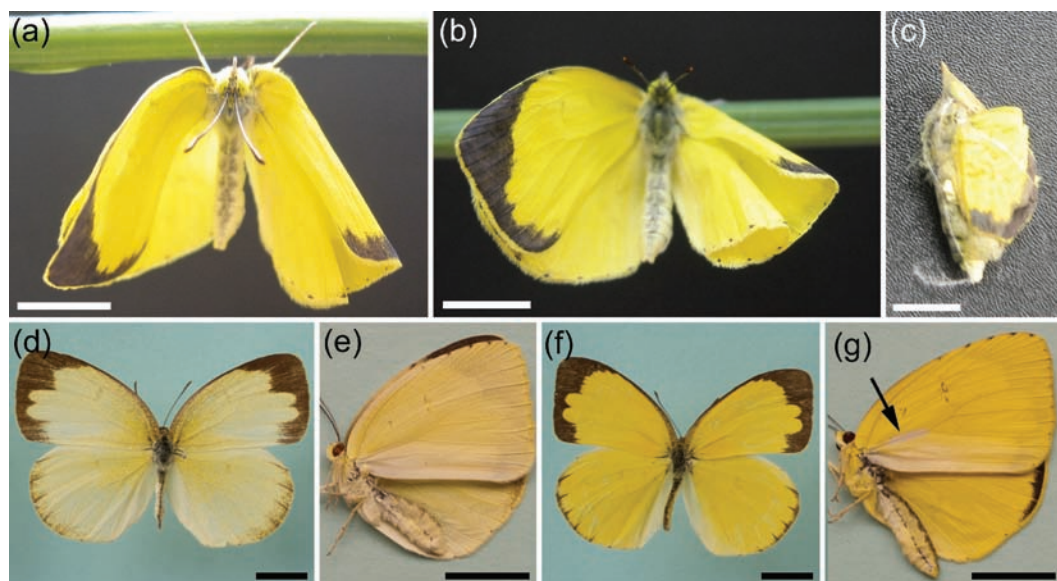


Color Figure 11.2 Bacterial functions in the medfly life cycle. This depiction shows known as well as possible interactions occurring between ovipositing female medflies (upper left), male medflies (upper right), medfly larvae (in the fruit), members of their associated bacterial community (green rods), other conspecific and heterospecific insects (lower left) and their own associated bacterial communities (red rods), and the fruit. Color legend for the mentioned functions, interactions, or mechanisms: green, demonstrated; purple, circumstantial evidence; blue, hypothetical (see main text for details).

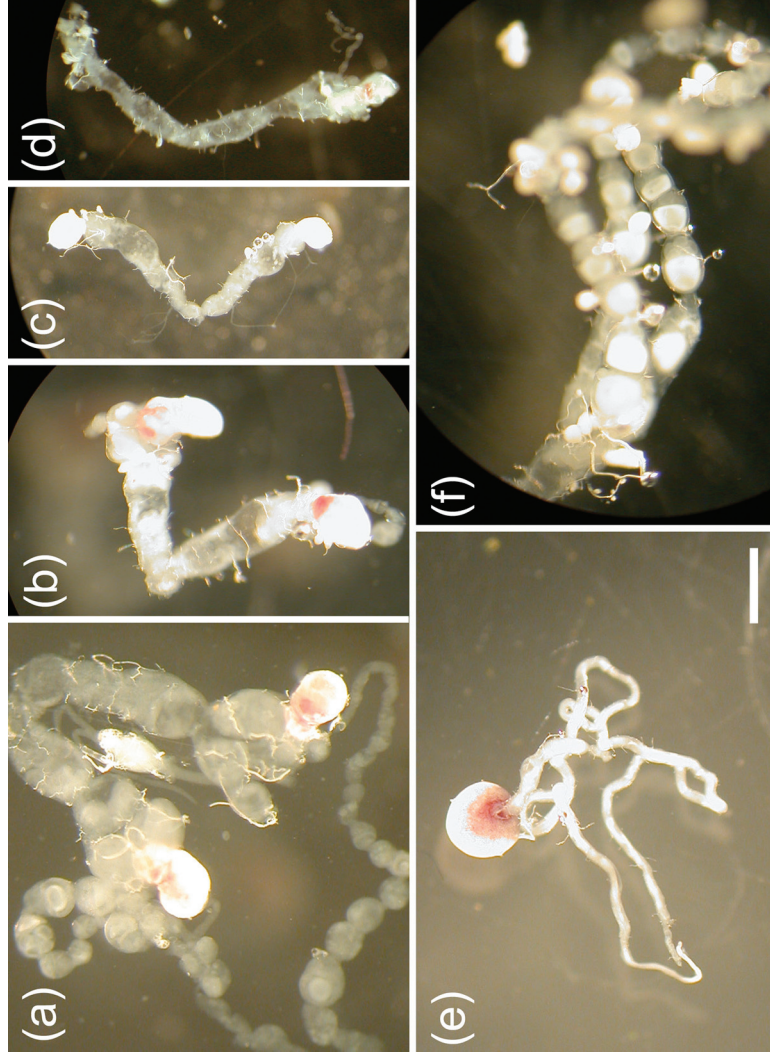


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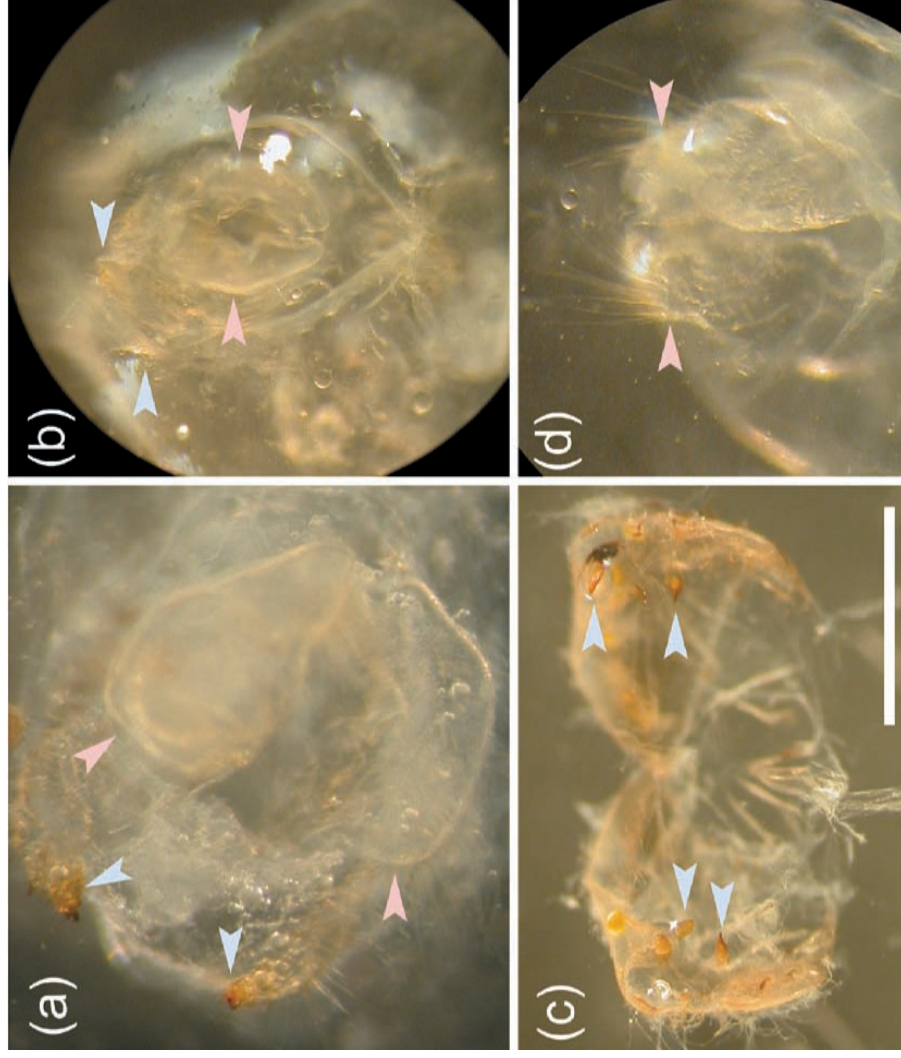
Color Figure 13.2 Different *Wolbachia* infection types and their phenotypes in *E. hecabe*. (a): In most populations, butterflies singly infected with *wHecCI* exhibit cytoplasmic incompatibility. (b): In populations from Okinawajima and Tanegashima, butterflies doubly infected with *wHecCI* and *wHecFem* exhibit feminization. (c): Butterflies singly infected with *wHecFem* have never been found in natural populations or in the laboratory. Right: A female adult of *E. hecabe* in the natural condition. (Photo provided by Dr. Masashi Nomura, Chiba University.)



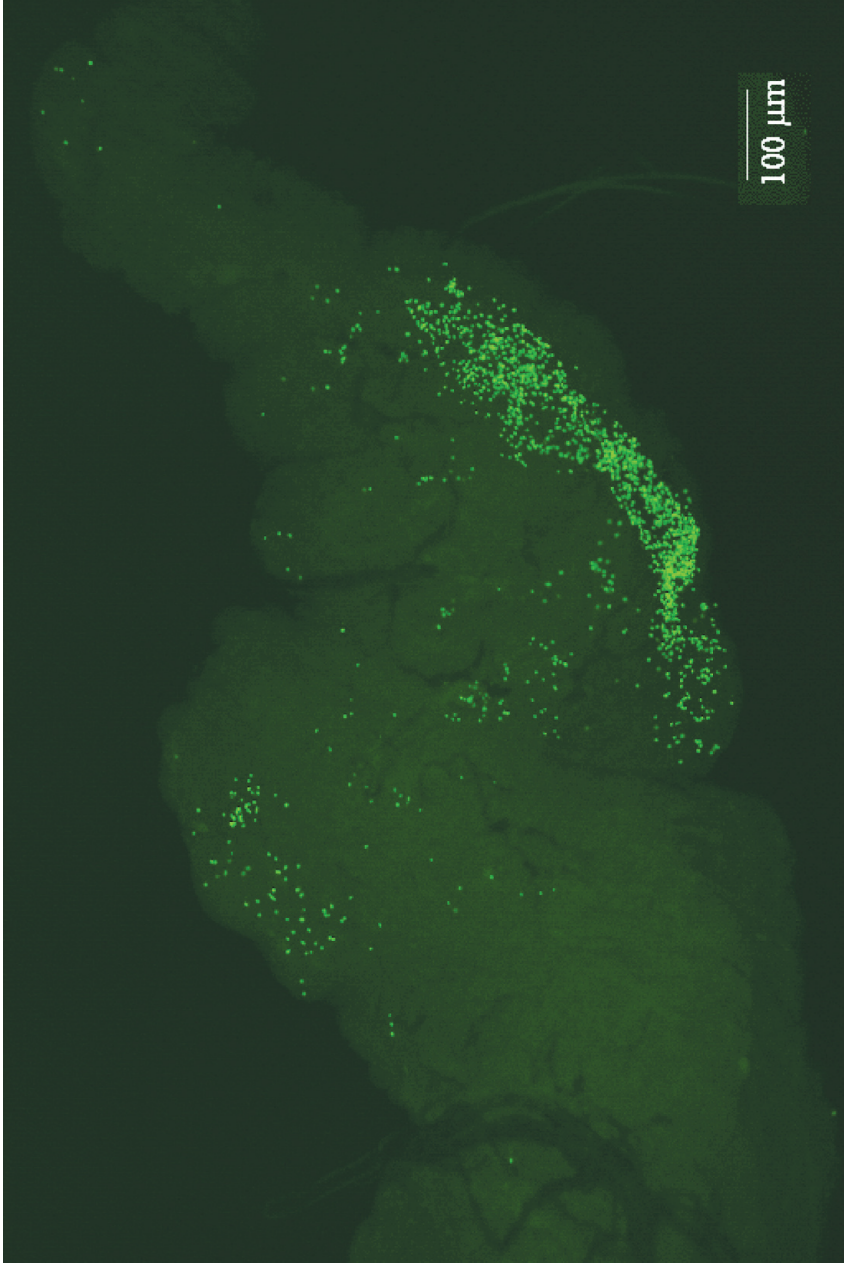
Color Figure 13.6 *E. hecabe* adults that emerged after larval antibiotic treatment. (a) and (b): Emerged adult insects with deformed wings obtained after antibiotic treatment from the third to fourth instar stages of an insect line doubly infected with *wHecCI* and *wHecFem*. (c): Adult insect that failed to escape from the pupal case obtained after antibiotic treatment from the first to fourth instar stages of an insect line doubly infected with *wHecCI* and *wHecFem*. (d) and (e): Normal adult females, pale in ground color and without sex brands, representing a nontreated insect line singly infected with *wHecCI*. (f) and (g): Normal adult males, bright in ground color and with sex brands (arrows), representing a nontreated insect line singly infected with *wHecCI*. Bars, 10 mm. (Adapted from Narita, S., Kageyama, D., Nomura, M., and Fukatsu, T. (2007a). *Appl. Environ. Microbiol.* **73**: 4332–4341. With permission.)



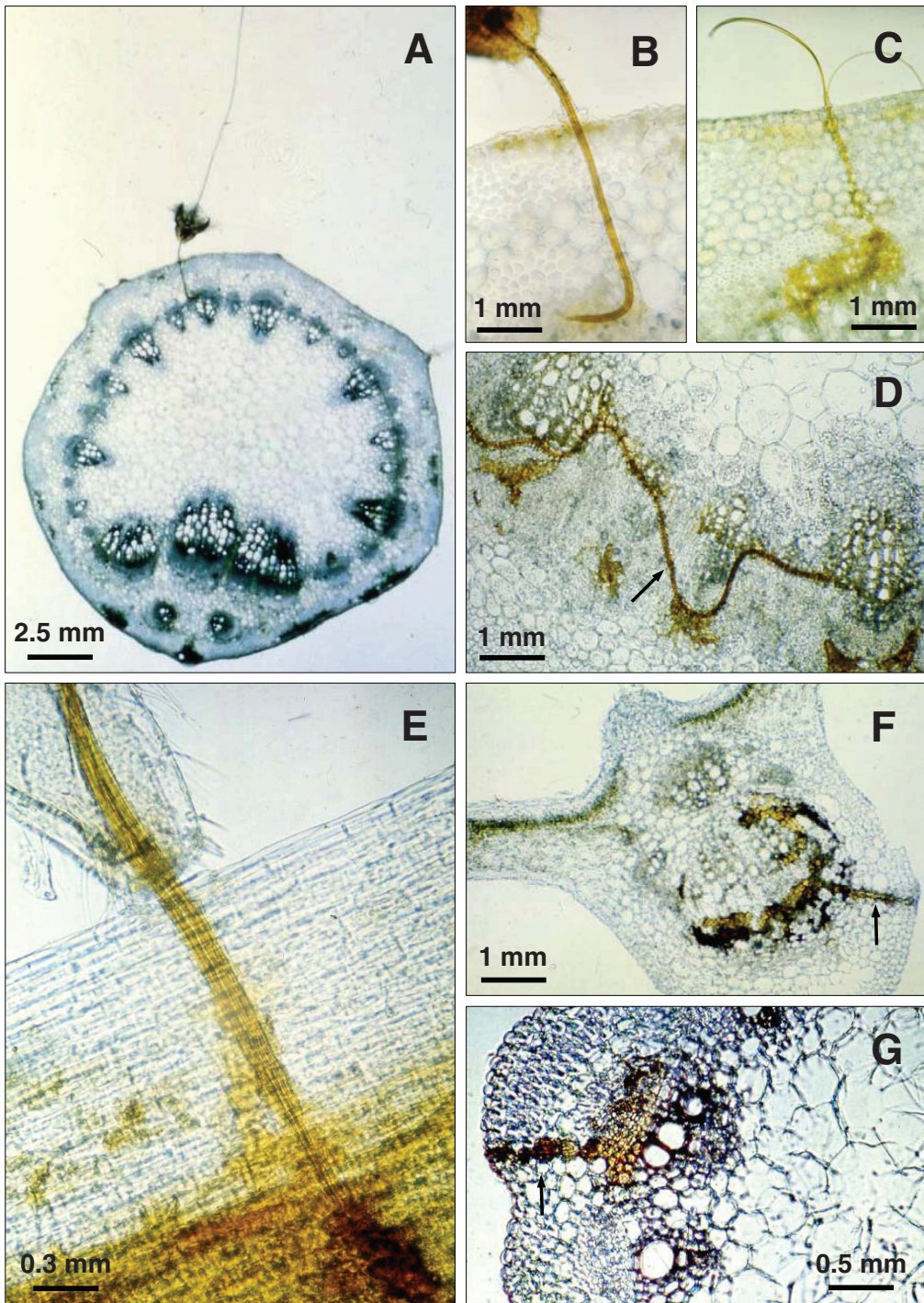
Color Figure 13.7 Reproductive organs of *E. hecabe* adults that emerged after larval antibiotic treatment. (a): Two deformed testes coexisting with a mature ovary obtained after antibiotic treatment from the first to fourth instar stages of an insect line doubly infected with *wHecCI* and *wHecFem*. (b) and (c): Two deformed testes obtained after antibiotic treatment from the first to fourth instar stages of an insect line doubly infected with *wHecCI* and *wHecFem*. (d): A deformed testis obtained after antibiotic treatment from the first to fourth instar stages of an insect line doubly infected with *wHecCI* and *wHecFem*. (e): A normal testis from a nontreated insect line singly infected with *wHecCI*. Note that a *wHecCI*. (f): A normal ovary from a nontreated insect line singly infected with *wHecCI*. Note that a pair of testes are often fused into one testis in lepidopteran adult insects. Arrows indicate testes. Bar, 1 mm. (Adapted from Narita, S., Kageyama, D., Nomura, M., and Fukatsu, T. (2007a). *Appl. Environ. Microbiol.* **73**: 4332–4341. With permission.)



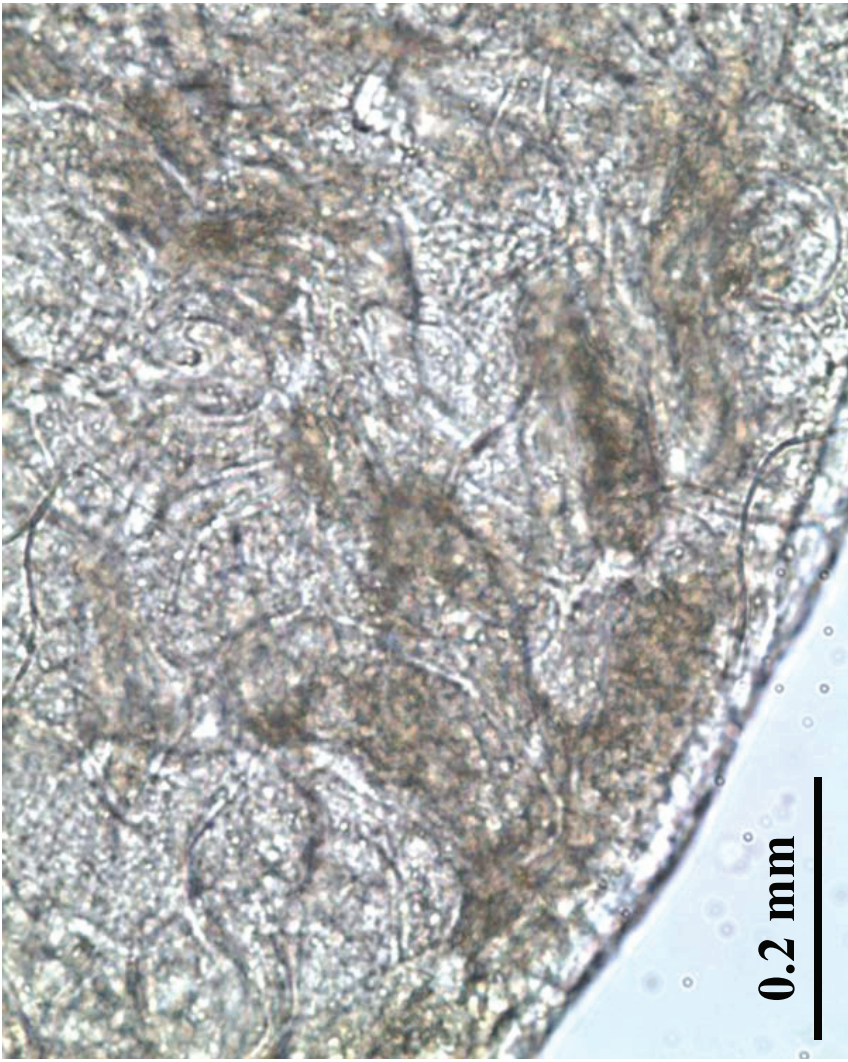
Color Figure 13.8 Genitalia preparations of *E. hecabe* adults that emerged after larval antibiotic treatment. (a) and (b): Sexually intermediate genitalia obtained after antibiotic treatment from the first to fourth instar stages of an insect line doubly infected with *wHecCI* and *wHecFem*. (c): Male genitalia from a nontreated insect line singly infected with *wHecCI*. (d): Female genitalia from a nontreated insect line singly infected with *wHecCI*. Blue arrowheads indicate male traits (*bicuspid apex of valva*), and pink arrowheads indicate female traits (*papilla analis*). Bar, 1 mm. (Adapted from Narita, S., Kageyama, D., Nomura, M., and Fukatsu, T. (2007a). *Appl. Environ. Microbiol.* 73: 4332–4341. With permission.)



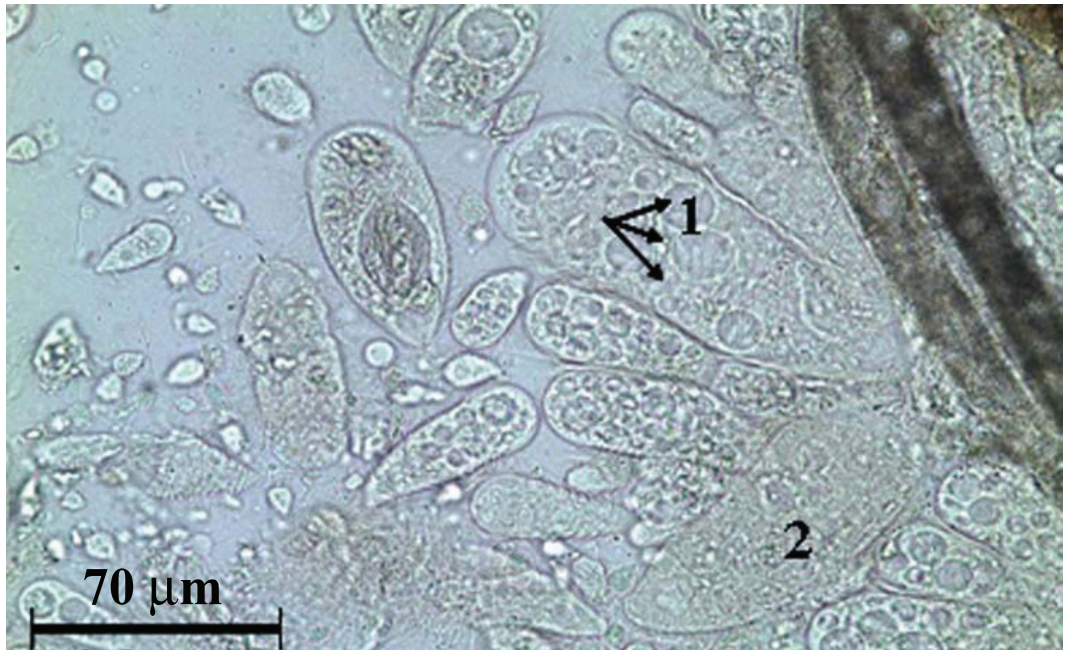
Color Figure 15.3 The gut of a female *Anopheles stephensi* mosquito, colonized by transformed bacteria of the genus *Asaia* that express the green fluorescent protein (GFP). A massive colonization is located at the midgut level.



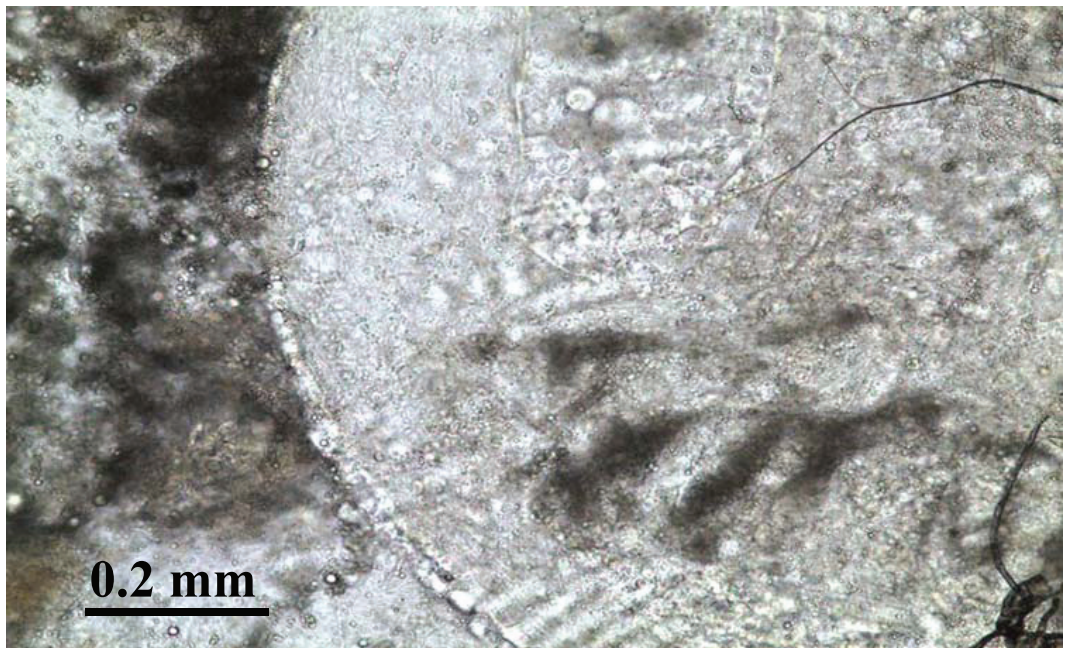
Color Figure 16.1 Details of stylets of phloem sucking insects and grapevine leaf tissues during biting. (A–D) *Peryceria purchasi* stylet penetrating grapevine tissues. (A–C) The pictures show the plasticity of the stylet that is inserted between cells in the parenchymatic tissue to reach the phloem cells. (D) Details of the stylet track (arrow) within the leaf tissue showing that the stylet of *P. purchasi* has explored different phloem tubes. (E–G) Biting of grapevine tissues by *S. titanus*. (E) The stylet of *S. titanus* penetrating the leaf tissues. (F and G) Tracks (arrows) left by the stylet of *S. titanus* in the vein of grapevine leaves



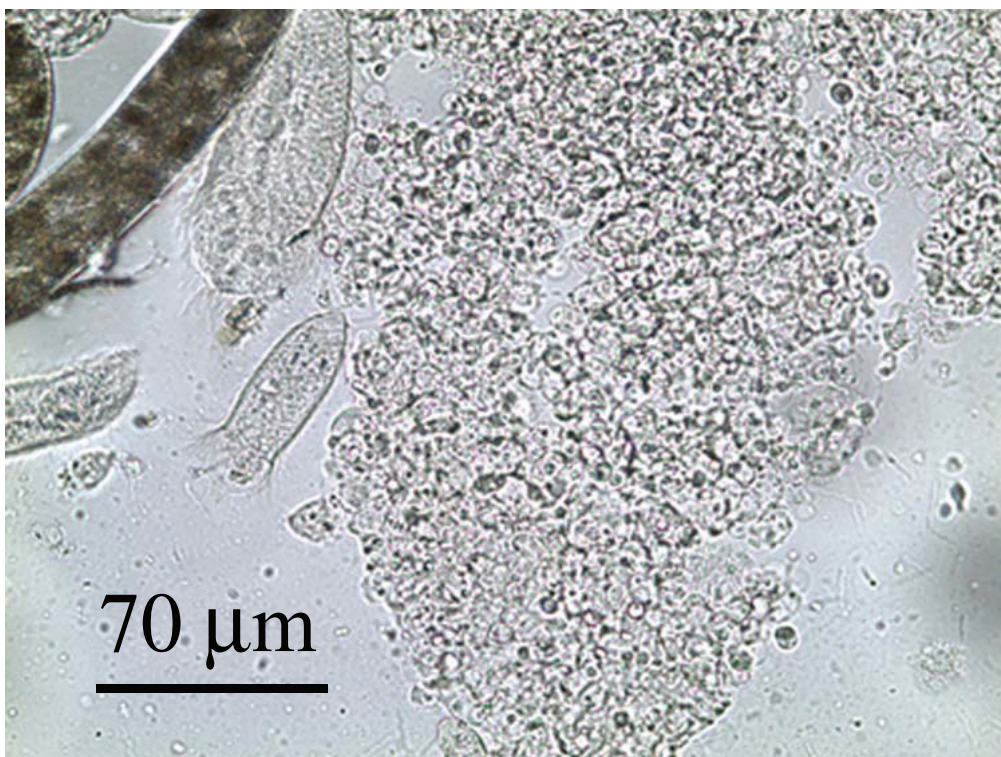
Color Figure 17.1 Healthy termite gut containing a dense protozoa population.



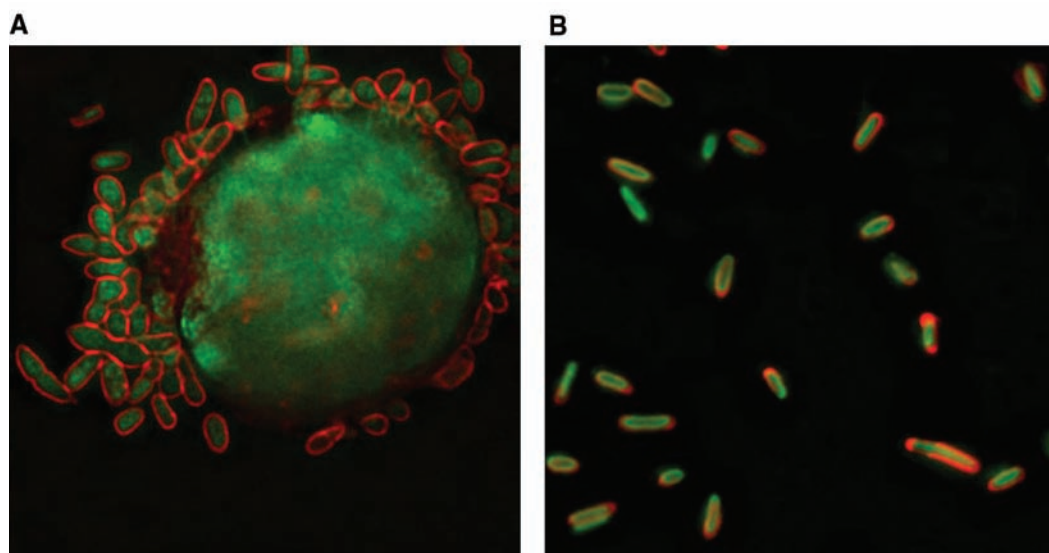
Color Figure 17.2a Deterioration of protozoa in the hindgut after workers were fed *D-Hecate*. 1 = vesicles inside affected protozoan. 2 = dead protozoan.



Color Figure 17.2b Defaunated hindgut.



Color Figure 17.3 Defaunation of worker hindgut after injection of lytic peptides.



Color Figure 18.2 Symbionts in insect cell culture and pure culture. Insect cell lines are useful for the culture of facultative symbionts and the study of interactions between symbionts and host cells. In plate A, *Ca. Arsenophonus arthropodicus* is attached to the surface of an *Aedes albopictus* C6/36 cell. The insect cell and bacteria were fixed and stained with FM4-64 (which binds to cellular lipids) and DAPI (which binds to nucleic acids) and visualized by deconvolution fluorescence microscopy. In plate B, live bacterial cells from a pure culture of *S. glossinidius* were visualized by fluorescence microscopy following staining with FM4-64 and DAPI. Pure culture isolation provides opportunities for the genetic manipulation of facultative symbionts.

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